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PRINCIPAL INVESTIGATOR: David Feldman, M.D.

CONTRACTING ORGANIZATION: Stanford University
Stanford, California 94305-5401

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13. ABSTRACT (Maximum 200 Words) The purpose of this research is to gain an understanding of the vitamin D - androgen interaction in prostate cancer. Calcitriol, the active hormonal form of vitamin D, induces androgen receptors (AR) in prostate cells. Major findings thus far involve studies in two areas. First, the ability of vitamin D and retinoids to regulate AR levels in LNCaP human prostate cancer cells. These studies have led to a publication which is included in the Appendix. The major conclusion is that the growth inhibitory activity of vitamin D and retinoid hormones is androgen-dependent and the activity can be blocked by the AR antagonist, Casodex. Second, is a study of prostate cancer cells derived from a bone metastasis in a patient whose prostate cancer had progressed to become androgen-independent. As detailed in a paper that is in press, the cells have a double mutation in the AR. Further study of these cells is in progress in an attempt to determine the nature of the changes that led to androgen-independence. The significance of this work is that it will increase our understanding of factors that stimulate prostate cancer growth and will attempt to develop mechanisms to inhibit the growth and progression of prostate cancer.					
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FOREWORD

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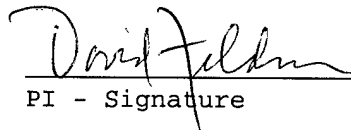
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Introduction

Calcitriol, the active hormonal form of Vitamin D, acts as a transcriptional regulator of target genes by interacting with a nuclear receptor known as the vitamin D receptor (VDR). The presence of VDR has been demonstrated in prostate cells, both primary cultures of normal prostate as well as in prostate cancer cell lines [1-4]. Calcitriol, acting via the VDR, inhibits prostate cancer cell growth as well as induces a number of target genes [4-8]. Of interest to this proposal, we have shown that calcitriol induces the androgen receptor (AR) in prostate cancer cells [9]. The basis of this grant is to investigate the role of vitamin D in prostate cancer with a focus on its role to increase AR abundance. We are pursuing several questions regarding the vitamin D - androgen interaction as they might impact on a number of aspects of prostate cancer biology: 1) androgen responsiveness, 2) effect of vitamin D on androgen ablation therapy, 3) the development of androgen-independence, and 4) the vitamin D axis as a determinant of prostate cancer risk.

Body

1. Liarozole acts synergistically with $1\alpha,25$ -dihydroxy-vitamin D₃ to inhibit growth of DU 145 human prostate cancer cells by blocking 24-hydroxylase.

Lan H. Ly, Xiao-Yan Zhao, Leah Holloway, and David Feldman

Endocrinology 140:2071-2076, 1999. This publication is in the Appendix.

$1\alpha,25$ -dihydroxyvitamin D₃ [$1,25-(OH)_2D_3$] inhibits the proliferation of many cancer cells in culture but not the aggressive human prostate cancer cell line DU 145. We postulated that the $1,25-(OH)_2D_3$ -resistant phenotype in DU 145 cells might result from the high levels of expression of 25-hydroxyvitamin D-24-hydroxylase (24-hydroxylase) induced by treatment with $1,25-(OH)_2D_3$. Since this P450 enzyme initiates $1,25-(OH)_2D_3$ catabolism, we presumed a high level of enzyme induction could limit the effectiveness of the $1,25-(OH)_2D_3$ antiproliferative action. To examine this hypothesis we explored combination therapy with liarozole fumarate (R85,246), an imidazole derivative currently in trials for prostate cancer therapy. Since imidazole derivatives are known to inhibit P450 enzymes, we postulated that this drug would inhibit 24-hydroxylase activity, increasing $1,25-(OH)_2D_3$ half-life, thereby enhancing $1,25-(OH)_2D_3$ antiproliferative effects on DU 145 cells. Cell growth was assessed by measurement of viable cells using the MTS assay. Neither $1,25-(OH)_2D_3$ (1-10 nM) nor liarozole (1-10 μ M) inhibited DU 145 cell growth when given alone. However, together at 4 days, $1,25-(OH)_2D_3$ (10 nM) and liarozole (1 μ M) inhibited growth 65%. We used a thin layer chromatography method to assess 24-hydroxylase activity and demonstrated that liarozole (1-100 μ M) inhibited this P450 enzyme in a dose-dependent manner. Moreover, liarozole treatment caused a significant increase in $1,25-(OH)_2D_3$ half-life from 11 to 31 h. In addition, $1,25-(OH)_2D_3$ can cause homologous up-regulation of the VDR and in the presence of liarozole, this effect was

amplified thus enhancing 1,25-(OH)₂D₃ activity. Western blot analyses demonstrated that DU 145 cells treated with 1,25-(OH)₂D₃/liarozole showed greater VDR up-regulation than with either drug alone. In summary, our data demonstrate that liarozole augments the ability of 1,25-(OH)₂D₃ to inhibit DU 145 cell growth. The mechanism appears to be due to inhibition of 24-hydroxylase activity leading to increased 1,25-(OH)₂D₃ half-life and augmentation of homologous up-regulation of VDR. We raise the possibility that combination therapy using 1,25-(OH)₂D₃ and liarozole or other inhibitors of 24-hydroxylase, both in non-toxic doses, might serve as an effective treatment for prostate cancer.

2. Induction of Androgen Receptor by 1 α , 25-Dihydroxyvitamin D₃ and 9-cis Retinoic Acid in LNCaP Human Prostate Cancer Cells

by: Xiao-Yan Zhao, Lan H. Ly, Donna M. Peehl, And David Feldman

Endocrinology 140: 1205-1212, 1999. This publication is included in the appendix.

The study is based on our recent findings that 1,25-(OH)₂D₃ inhibits proliferation of LNCaP cells, an androgen-responsive human prostate cancer cell line. Also, 1,25-(OH)₂D₃ increases androgen receptor (AR) abundance and enhances cellular responses to androgen in these cells. In the current study, we have investigated the mechanism by which 1,25-(OH)₂D₃ regulates AR gene expression and the involvement of AR in the 1,25-(OH)₂D₃- and 9-cis retinoic acid (RA)- mediated growth inhibition of LNCaP. Northern blot analyses demonstrated that the steady-state mRNA level of AR was significantly increased by 1,25-(OH)₂D₃ in a dose-dependent manner. Time course experiments revealed that the increase of AR mRNA by 1,25-(OH)₂D₃ exhibited delayed kinetics. In response to 1,25-(OH)₂D₃, AR mRNA levels were first detected to rise at 8 h and reached a maximal induction of 10-fold over the untreated control at 48 h; the effect was sustained at 72 h. Furthermore, the induction of AR mRNA by 1,25-(OH)₂D₃ was completely abolished by incubation of cells with cycloheximide, a protein synthesis inhibitor. 1,25-

(OH)₂D₃ was unable to induce expression of an AR promoter-luciferase reporter. These findings indicate that the stimulatory effect of 1,25-(OH)₂D₃ on AR gene expression was indirect. Western blot analyses showed an increase of AR protein in 1,25-(OH)₂D₃-treated cells. This increased expression of AR was followed by an inhibition of growth in LNCaP cells by 1,25-(OH)₂D₃. Similar to 1,25-(OH)₂D₃, 9-cis RA also induced AR mRNA expression and the effect of both hormones was additive. Moreover, 1,25-(OH)₂D₃ and 9-cis RA acted synergistically to inhibit LNCaP cell growth. These anti-proliferative effects of 1,25-(OH)₂D₃ and 9-cis RA alone or in combination were blocked by the pure AR antagonist, Casodex. In conclusion, our results demonstrate that growth inhibition of LNCaP by 1,25-(OH)₂D₃ and 9-cis RA is mediated by an AR-dependent mechanism and preceded by the induction of AR gene expression. This finding that differentiating agents such as vitamin D and A derivatives are potent inducers of AR may have clinical implications in the treatment of prostate cancer.

3. Two Mutations Identified In The Androgen Receptor Of A New Human Prostate Cancer Cell Line MDA PCa 2a

by: Xiao-Yan Zhao, Bryan Boyle, Aruna V. Krishnan, Nora M. Navone,
Donna M. Peehl, And David Feldman

In this study, we have characterized the androgen receptor (AR) in a new human prostate cancer cell line, MDA PCa 2a, that has recently been established from a bone metastasis of a patient whose cancer was exhibiting androgen-independent growth. These cells express abundant AR (N_{max}=685±149 fmol/mg protein), as determined by equilibrium binding assays with [³H]dihydrotestosterone (DHT). However, Scatchard analyses show the AR binding affinity for DHT in these cells to be only 25 nM, a 50-fold lower affinity than those of the mutated AR in LNCaP cells (K_d=0.5 nM) or the wildtype AR in MCF-7 cells (K_d=0.4 nM). DNA sequence analyses of the AR gene in MDA PCa 2a cells revealed two mutations in the ligand-binding domain, L701H and T877A, the latter

being reported previously in LNCaP cells. Compared to LNCaP, the new cell line is 10 to 1000-fold less responsive to androgens in cell growth assays as well as in stimulation of PSA secretion. Interestingly, in the absence of added androgens, the new cell line expresses 15-fold higher baseline levels of PSA than LNCaP, suggesting constitutive expression of its PSA gene. In summary, we have identified two mutations in the AR gene of the MDA PCa 2a cell line that are likely responsible for decreased binding affinity for DHT and partial androgen insensitivity observed in these cells. Both androgen insensitivity and elevated baseline PSA levels exemplify the androgen-independent phenotype. Thus, this new cell line can serve as a functionally relevant model system of advanced prostate cancer and can be used to study important events related to androgen-independent growth.

Key Research Accomplishments

1. AR expression is increased in prostate cancer cells by vitamin D and retinoids.
2. The ability of vitamin D and retinoids to inhibit LNCaP cell growth is AR-dependent.
3. The MDA PCa 2A cell line has two mutations in the ligand binding domain of the AR which reduce its affinity for androgens.

Reportable Outcomes

Manuscripts

1. Liarozole acts synergistically with $1\alpha,25$ -dihydroxy-vitamin D₃ to inhibit growth of DU 145 human prostate cancer cells by blocking 24-hydroxylase

Lan H. Ly , Xiao-Yan Zhao, Leah Holloway, and David Feldman

2. Induction of Androgen Receptor by $1\alpha, 25$ -Dihydroxyvitamin D₃ and 9-cis Retinoic Acid in LNCaP Human Prostate Cancer Cells

by: Xiao-Yan Zhao, Lan H. Ly, Donna M. Peehl, And David Feldman

Endocrinology 140:1205-1212, 1999

3. Two Mutations Identified In The Androgen Receptor Of A New Human Prostate Cancer Cell Line MDA PCa 2a

by: Xiao-Yan Zhao, Bryan Boyle, Aruna V. Krishnan, Nora M. Navone,

Donna M. Peehl, And David Feldman

Journal of Urology, In press

Abstracts

1. Liarozole acts synergistically with $1\alpha,25$ -dihydroxy-vitamin D₃ to inhibit growth of DU 145 human prostate cancer cells by blocking 24-hydroxylase

Xiao-Yan Zhao, Lan H. Ly, Leah Holloway, and David Feldman

Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305

Amer Soc Bone & Mineral Research, Dec 1-6, 1998 San Francisco, Pg. S265, abstract T267

2. Androgen insensitivity due to a double mutation in the androgen receptor of a new human prostate cancer cell line MDA PCa 2a Xiao-Yan Zhao¹, Bryan Boyle¹, Aruna V. Krishnan¹, Peter J. Malloy¹, Nora M. Navone³, Donna M. Peehl², and David Feldman¹. Departments of ¹Medicine and ²Urology, Stanford University, Palo Alto, CA 94305 and ³Department of GU Medical Oncology, M.D. Anderson Cancer Center, Houston, TX 77030

Endocrine Society 81st Annual Meeting, June 12-15, 1998, San Diego, CA. Pg 264, Abstract P1-613

Conclusions

The role of vitamin D in the prostate is complex and interesting. Vitamin D regulates AR abundance altering the cellular response to androgens. The vitamin D-androgen axis appears to be important in the progression from androgen-dependent to androgen-independent growth. Further studies are warranted to elucidate various aspects of the hormonal regulation of prostate cancer cells in the hopes of developing new therapeutic strategies to treat this common malignancy of men.

References

1. Skowronski RJ, Peehl DM, Feldman D: Vitamin D and prostate cancer: 1,25 dihydroxyvitamin D₃ receptors and actions in human prostate cancer cell lines. *Endocrinology* 1993; 132(5): 1952-60.
2. Peehl DM, Skowronski RJ, Leung GK, Wong ST, Stamey TA, Feldman D: Antiproliferative effects of 1,25-dihydroxyvitamin D₃ on primary cultures of human prostatic cells. *Cancer Res* 1994; 54(3): 805-10.
3. Miller GJ, Stapleton GE, Ferrara JA, et al.: The human prostatic carcinoma cell line LNCaP expresses biologically active, specific receptors for 1 α ,25-dihydroxyvitamin D₃. *Cancer Research* 1992; 52: 515-520.
4. Miller GJ, Stapleton GE, Hedlund TE, Moffatt KA: Vitamin D receptor expression, 24-hydroxylase activity, and inhibition of growth by 1 α ,25-dihydroxyvitamin D₃ in seven human prostatic carcinoma cell lines. *Clinical Cancer Research* 1995; 1: 997-1003.
5. Skowronski RJ, Peehl DM, Feldman D: Actions of vitamin D₃, analogs on human prostate cancer cell lines: comparison with 1,25-dihydroxyvitamin D₃. *Endocrinology* 1995; 136(1): 20-6.
6. Schwartz GG, Hill CC, Oeler TA, Becich MJ, Bahnson RR: 1,25-Dihydroxy-16-ene-23-yne-vitamin D₃ and prostate cancer cell proliferation *in vivo*. *Urology* 1995; 46(3): 365-9.
7. Zhuang SH, Burnstein KL: Antiproliferative effect of 1 α ,25-dihydroxyvitamin D₃ in human prostate cancer cell line LNCaP involves reduction of cyclin-dependent kinase 2 activity and persistent G1 accumulation. *Endocrinology* 1998; 139(3): 1197-207.
8. Blutt SE, Allegretto EA, Pike JW, Weigel NL: 1,25-dihydroxyvitamin D₃ and 9-cis-retinoic acid act synergistically to inhibit the growth of LNCaP prostate cells and cause accumulation of cells in G1. *Endocrinology* 1997; 138(4): 1491-7.
9. Zhao XY, Ly LH, Peehl DM, Feldman D: 1 α ,25-dihydroxyvitamin D₃ actions in LNCaP human prostate cancer cells are androgen-dependent. *Endocrinology* 1997; 138(8): 3290-8.

Appendicies

1. Liarozole acts synergistically with $1\alpha,25$ -dihydroxy-vitamin D3 to inhibit growth of DU 145 human prostate cancer cells by blocking 24-hydroxylase.

Lan H. Ly, Xiao-Yan Zhao, Leah Holloway, and David Feldman
Endocrinology 140:2071-2076, 1999.

2. Induction of Androgen Receptor by $1\alpha, 25$ -Dihydroxyvitamin D₃ and 9-cis Retinoic Acid in LNCaP Human Prostate Cancer Cells

by: Xiao-Yan Zhao, Lan H. Ly, Donna M. Peehl, And David Feldman
Endocrinology 140: 1205-1212, 1999.

3. Androgen insensitivity due to a double mutation in the Androgen Receptor Of A New Human Prostate Cancer Cell Line MDA PCa 2a (Abstract)

by: Xiao-Yan Zhao, Bryan Boyle, Aruna V. Krishnan, Nora M. Navone,
Donna M. Peehl, And David Feldman

81st Annual Meeting of the Endocrine Society, June 12-15, 1999.

San Diego, CA. Abstract P1-613

Liarozole Acts Synergistically with 1 α ,25-Dihydroxyvitamin D₃ to Inhibit Growth of DU 145 Human Prostate Cancer Cells by Blocking 24-Hydroxylase Activity*

LAN H. LY, XIAO-YAN ZHAO, LEAH HOLLOWAY, AND DAVID FELDMAN

Department of Medicine, Division of Endocrinology, Stanford University School of Medicine, Stanford, California 94305

ABSTRACT

1 α ,25-Dihydroxyvitamin D₃ [1,25-(OH)₂D₃] inhibits the proliferation of many cancer cells in culture, but not the aggressive human prostate cancer cell line DU 145. We postulated that the 1,25-(OH)₂D₃-resistant phenotype in DU 145 cells might result from the high levels of expression of 25-hydroxyvitamin D-24-hydroxylase (24-hydroxylase) induced by treatment with 1,25-(OH)₂D₃. As this P450 enzyme initiates 1,25-(OH)₂D₃ catabolism, we presumed that a high level of enzyme induction could limit the effectiveness of the 1,25-(OH)₂D₃ antiproliferative action. To examine this hypothesis we explored combination therapy with liarozole fumarate (R85,246), an imidazole derivative currently in trials for prostate cancer therapy. As imidazole derivatives are known to inhibit P450 enzymes, we postulated that this drug would inhibit 24-hydroxylase activity, increasing the 1,25-(OH)₂D₃ half-life, thereby enhancing 1,25-(OH)₂D₃ antiproliferative effects on DU 145 cells. Cell growth was assessed by measurement of viable cells using the MTS assay. When used alone, neither 1,25-(OH)₂D₃ (1–10 nM) nor liarozole (1–10 μ M) inhibited DU 145 cell growth. However, when added together, 1,25-(OH)₂D₃ (10

nM)/liarozole (1 μ M) inhibited growth 65% after 4 days of culture. We used a TLC method to assess 24-hydroxylase activity and demonstrated that liarozole (1–100 μ M) inhibited this P450 enzyme in a dose-dependent manner. Moreover, liarozole treatment caused a significant increase in 1,25-(OH)₂D₃ half-life from 11 to 31 h. In addition, 1,25-(OH)₂D₃ can cause homologous up-regulation of the vitamin D receptor (VDR), and in the presence of liarozole, this effect was amplified, thus enhancing 1,25-(OH)₂D₃ activity. Western blot analyses demonstrated that DU 145 cells treated with 1,25-(OH)₂D₃/liarozole showed greater VDR up-regulation than cells treated with either drug alone. In summary, our data demonstrate that liarozole augments the ability of 1,25-(OH)₂D₃ to inhibit DU 145 cell growth. The mechanism appears to be due to inhibition of 24-hydroxylase activity, leading to increased 1,25-(OH)₂D₃ half-life and augmentation of homologous up-regulation of VDR. We raise the possibility that combination therapy using 1,25-(OH)₂D₃ and liarozole or other inhibitors of 24-hydroxylase, both in nontoxic doses, might serve as an effective treatment for prostate cancer. (*Endocrinology* 140: 2071–2076, 1999)

THE MAJOR biological action of 1 α ,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃], the active metabolite of vitamin D, is to maintain calcium homeostasis in the body (1). Recent findings indicate that 1,25-(OH)₂D₃ is also involved in regulating cellular proliferation and differentiation in various target tissues that possess vitamin D receptors (VDR) (1–4). 1,25-(OH)₂D₃ and less calcemic analogs have been shown to inhibit cell growth in both human prostate carcinoma cell lines (5–11) and primary cultures of normal and prostate cancers (12). However, 1,25-(OH)₂D₃ showed only minimal inhibition of cell proliferation of DU 145, a human prostate cancer cell line derived from a brain metastasis, despite the presence of substantial amounts of VDR in this cell type (5, 7). The mechanism for the relative unresponsiveness of DU 145 to the antiproliferative action of 1,25-(OH)₂D₃ is not known.

DU 145 cells have been shown to express high levels of

25-hydroxyvitamin D-24-hydroxylase (24-hydroxylase) after treatment with 1,25-(OH)₂D₃ (5, 7). LNCaP cells can be induced to express low levels of 24-hydroxylase activity [$12.6 \pm 3.1 \times 10^{-9}$ μ mol/2 $\times 10^6$ cells/30 min of 24,25(OH)₂D₃ produced] and are substantially growth inhibited by 1,25-(OH)₂D₃, whereas DU 145 cells can be induced to express very high levels of 24-hydroxylase activity ($96.7 \pm 39.5 \times 10^{-9}$ μ mol/2 $\times 10^6$ cells/30 min) and are minimally growth inhibited (5, 7). As this P450 enzyme initiates the 1,25-(OH)₂D₃ inactivation pathway (1), we (8) and others (7, 13) have considered the possibility that rapid breakdown of 1,25-(OH)₂D₃ by 24-hydroxylase might be the cause of the resistant phenotype in DU 145 cells. In this study, we examine the premise that combination treatment with 1,25-(OH)₂D₃ and an inhibitor of 24-hydroxylase might render DU 145 cells more sensitive to the antiproliferative action of 1,25-(OH)₂D₃.

Combination therapy is often used to enhance the anticancer activity of various agents. Ketoconazole, liarozole, and other inhibitors of P450 enzymes may exhibit anticancer properties via several pathways, including actions on critical enzyme pathways (13, 14). In this study, we examined the possibility that combination treatment with 1,25-(OH)₂D₃ and liarozole, an imidazole derivative with antiproliferative properties (15, 16), might result in enhanced growth inhibition of DU 145 cells. Liarozole is known to inhibit

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Address all correspondence and requests for reprints to: David Feldman, M.D., Stanford University School of Medicine, Stanford, California 94305-5103. E-mail: feldman@cmgm.stanford.edu.

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several cytochrome P-450 enzymes, including retinoic acid 4-hydroxylase and aromatase (15, 17, 18). It is suspected that the former activity prolongs the half-life of retinoic acid and thereby increases the antiproliferative activity of endogenous retinoic acid when liarazole is administered to patients with prostate cancer (15, 16, 18). Here we show that 1,25-(OH)₂D₃ and liarazole interact synergistically to inhibit DU 145 cell growth. Our data demonstrate, for the first time, the ability of liarazole to directly inhibit 24-hydroxylase activity. The mechanism of liarazole action on DU 145 cells appears to be via inhibition of 24-hydroxylase, which causes a dual effect to prolong 1,25-(OH)₂D₃ half-life and to enhance up-regulation of VDR levels. Additional mechanisms may also play a role.

Materials and Methods

Materials

25-Hydroxy-[³H]vitamin D₃ (SA, 12.7 Ci/mmol) was obtained from Amersham Chemical Co. (Arlington Heights, IL). Liarazole fumarate (5-[(3-chlorophenyl)(1H-imidazol-1-yl)methyl]1H-benzimidazole fumarate) was a gift from Dr. C. Bowden (Janssen Research Foundation, Spring House, PA), and 1,25-(OH)₂D₃ was a gift from Dr. M. Uskokovic (Hoffmann-LaRoche, Inc., Nutley, NJ). Aprotinin, pepstatin, and soybean trypsin inhibitor were purchased from Boehringer Mannheim (Indianapolis, IN). Tissue culture media were purchased from Mediatech (Herndon, VA). FBS was obtained from Life Technologies (Gaithersburg, MD). CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS reagent) was purchased from Promega Corp. (Madison, WI). Silica gel TLC plates were purchased from E. M. Science (Darmstadt, Germany). All other reagents, except where indicated, were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture

The DU 145 human prostate carcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were routinely cultured in RPMI 1640 medium supplemented with 5% FBS and antibiotics at 37 C in a humidified atmosphere of 5% CO₂.

Assay of cell growth

Cell growth was assessed by measurement of viable cells using the MTS assay. DU 145 cells were trypsinized and seeded at a density of approximately 2,000 cells/well in 96-well tissue culture plates (Falcon, Lincoln Park, NJ) in 200 μl culture medium. The cells were allowed to attach for 24 h, and the medium was replaced with fresh medium containing 5% FBS. Cells were then treated with vehicle (ethanol), 1,25-(OH)₂D₃, and/or liarazole. Triplicate wells were used for each experimental condition. The medium containing vehicle or test compounds was renewed every 2 days during the course of the experiment. After the appropriate incubation period, the cells were processed by replacing them with fresh RPMI 1640 medium containing MTS reagent (100 μl medium plus 20 μl MTS reagent/well). The plates were incubated at 37 C in a humidified atmosphere of 5% CO₂ for approximately 3–4 h. The absorbance at 490 nm was read using an automatic plate reader (Emax Precision Microplate Reader, Molecular Devices, Menlo Park, CA) and was linear up to the highest cell concentration tested (40,000 cells/well).

Induction of 24-hydroxylase activity

24-Hydroxylase enzyme activity was assayed in a cell suspension system slightly modified from the method previously described (19). A 100-mm² confluent DU 145 culture, growing under standard conditions, was treated for various times (0.5, 3, 6, 16, and 20 h) with either vehicle (ethanol) or 10 nM 1,25-(OH)₂D₃. Cells were then rinsed with 10 ml PBS and incubated with 10 ml culture medium at 37 C in a humidified atmosphere of 5% CO₂ for approximately 30 min to remove 1,25-(OH)₂D₃. Cells were then trypsinized and resuspended at 10⁶ cells/200 μl RPMI 1640 containing 10 mM HEPES with 1% FBS. The cells were

incubated for 30 min at 37 C with 1.0 nM [³H]25-OHD₃ and 1.0 μM 25-(OH)₂D₃. The reaction was terminated by the addition of 750 μl methanol-chloroform (2:1) and 20 μl 24,25-(OH)₂D₃. The metabolites were extracted three times with 200 μl chloroform. The organic extracts were combined, dried with a Speed-Vac (Savant Instruments, Farmingdale, NY) and dissolved in a 90:10 mixture of hexane-isopropanol. The production of [³H]24,25-(OH)₂D₃ was quantitated by TLC on silica gel/aluminum foil plates developed in methylene chloride-ethyl acetate (1:1) run with authentic standards. The TLC strips were cut into 14 fractions and placed individually in minicounting vials. This TLC system produced good separation of [³H]25-(OH)₂D₃ from [³H]24,25-(OH)₂D₃.

Inhibition of 24-hydroxylase activity

Time-course studies indicate that induction of 24-hydroxylase activity could be detected at 3 h by 10 nM 1,25-(OH)₂D₃ treatment with a plateau at approximately 20 h. Therefore, the conditions selected for studying the inhibition of 24-hydroxylase activity by liarazole were 20-h induction, 10⁶ cells, 1 nM [³H]25-(OH)₂D₃, and 30-min incubation with various concentrations of liarazole (1, 10, 50, and 100 μM).

Determination of 1,25-(OH)₂D₃ half-life

The half-life of 1,25-(OH)₂D₃ in DU 145 cells was determined by measuring the residual unmetabolized [³H]1,25-(OH)₂D₃ in the conditioned medium at various time points after addition. Confluent DU 145 cells were treated for various times with [³H]1,25-(OH)₂D₃ in the presence or absence of liarazole. Two hundred microliters of conditioned medium were mixed with 750 μl methanol-chloroform (2:1), and the metabolites were extracted with 200 μl chloroform. Chloroform extraction was repeated three times. The organic extracts were dried with a Speed-Vac and dissolved in a 90:10 mixture of hexane-isopropanol. The disappearance of [³H]1,25-(OH)₂D₃ and the production of [³H]1,24,25-(OH)₃D₃ were quantitated by a TLC system using methylene chloride-ethyl acetate (1:3). After 145 min of development, the TLC strips were dried and fractionated by cutting regions identified as 1,25-(OH)₂D₃ and 1,24,25-(OH)₃D₃ by comigration of authentic standards. This TLC system gave good separation between [³H]1,25-(OH)₂D₃ and [³H]1,24,25-(OH)₃D₃. The R_f value for 1,25-(OH)₂D₃ was 0.667, and that for 1,24,25-(OH)₃D₃ was 0.333.

Western blot analysis of vitamin D receptor (VDR)

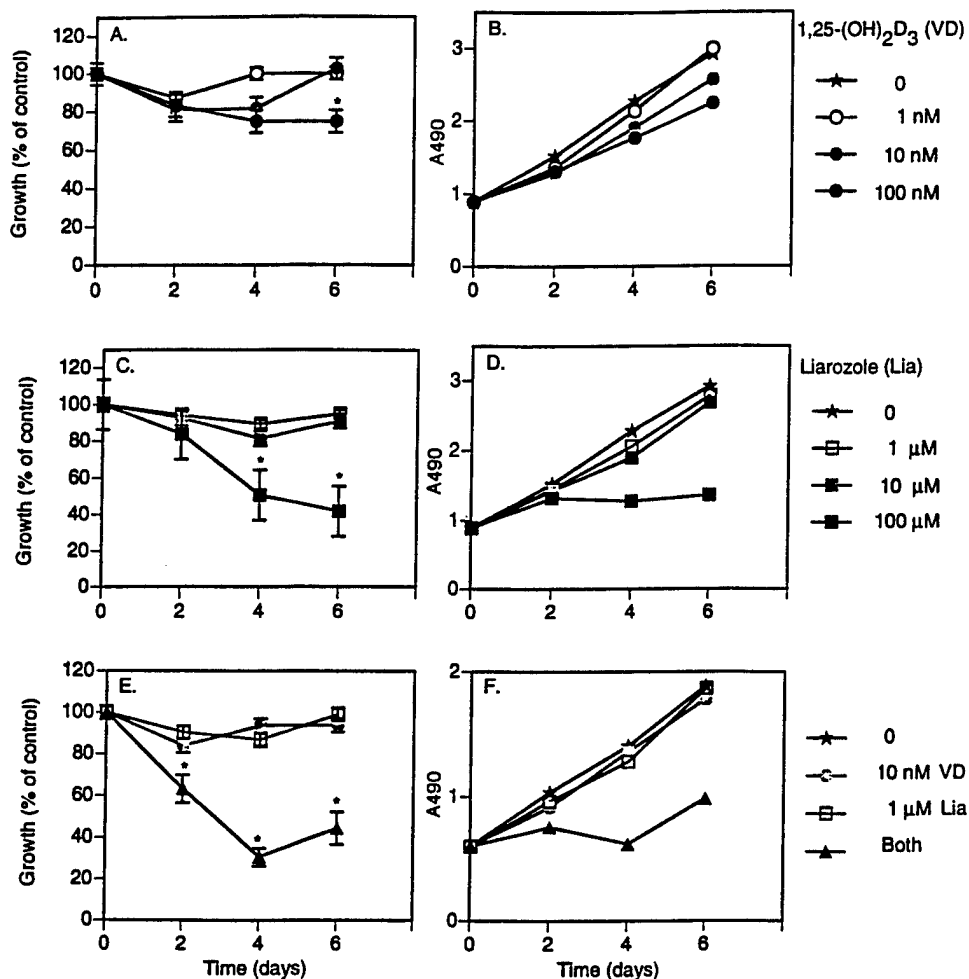
Cell monolayers grown in RPMI 1640 supplemented with 5% charcoal-stripped serum in 100-mm dishes were incubated with ethanol vehicle, 1,25-(OH)₂D₃ (0.1, 1, and 10 nM), and/or liarazole (10 μM) for 4 days. After 4 days of incubation, cells were harvested, and Western blot analysis was performed as described previously using anti-VDR monoclonal antibody (9A7) (20). The experiment was repeated twice with similar results.

Results

Combination effect of 1,25-(OH)₂D₃ and liarazole on DU 145 cell growth

DU 145 cells are only minimally responsive to the antiproliferative effect of 1,25-(OH)₂D₃ (5, 7, 8). In our current studies, DU 145 cells were treated with increasing concentrations of 1,25-(OH)₂D₃ (1, 10, and 100 nM) over a time course of 2, 4, and 6 days (Fig. 1, A and B). The growth of DU 145 cells was not significantly inhibited by the lower concentrations of 1 and 10 nM; however, at the highest concentration (100 nM), there was a slight growth inhibition of approximately 20% on day 6. Similarly, as shown in Fig. 1, C and D, liarazole failed to inhibit the proliferation of DU 145 cells at 1 and 10 μM, but 100 μM resulted in 50% growth inhibition at 4 days and 60% growth inhibition at 6 days. However, 100 μM liarazole is a toxic dose and when administered to patients at these concentrations it causes hypervi-

FIG. 1. Dose-response effect of 1,25-(OH)₂D₃, liarzozole, and the combination on DU 145 cell growth over a time course of 6 days. Cells were plated at approximately 2000 cells/well in 96-well tissue culture plates in 200 μ l medium with the indicated concentrations of hormone. Media were changed every 2 days. Cell proliferation was estimated using the MTS assay. Data are expressed as the mean \pm SD ($n = 3$) in the left panels. The right panels show a single representative experiment comparing treatment to vehicle and expressed in absorbance units. *, Significant changes ($P < 0.05$) compared with the ethanol control. A and B, Treatment with 1,25-(OH)₂D₃. C and D, Treatment with liarzozole. E and F, Treatment with a combination of 1,25-(OH)₂D₃ (10 nM) and liarzozole (1 μ M).



taminosis A. Neither 10 nM 1,25-(OH)₂D₃ nor 1 μ M liarzozole had any antiproliferative effect when used alone. However as shown in Fig. 1, E and F, the combination treatment caused 60% growth inhibition. These data indicate that 1,25-(OH)₂D₃ and liarzozole interact synergistically to inhibit DU 145 cell growth.

Inhibition of 24-hydroxylase activity by liarzozole

We next investigated the possible mechanisms by which liarzozole enhanced the ability of 1,25-(OH)₂D₃ to inhibit DU 145 cell proliferation. As shown in many other cell culture systems, we found here that 1,25-(OH)₂D₃ induced 24-hydroxylase activity in DU 145 cells in a time-dependent manner (Fig. 2). The level of 24-hydroxylase activity in DU 145 cells is much higher than that in other cell types, particularly compared with LNCaP cells, which are substantially inhibited by 1,25-(OH)₂D₃ alone (5, 7). Liarzozole had no intrinsic ability to induce 24-hydroxylase activity. However, liarzozole can inhibit 24-hydroxylase activity. After treating cells with 10 nM 1,25-(OH)₂D₃ for 20 h to induce 24-hydroxylase activity, we examined the abilities of various concentrations of liarzozole to inhibit enzyme activity by blocking the conversion of [³H]25-OHD₃ to 24,25-(OH)₂D₃. As shown in Fig. 3, liarzozole (1, 10, 50, and 100 μ M) was able to directly inhibit 24-hydroxylase activity in a dose-dependent manner, such

that 10 μ M liarzozole resulted in approximately 80% inhibition of enzyme activity compared with the activity of the induced cells in the absence of liarzozole.

Effect of liarzozole on 1,25-(OH)₂D₃ half-life

As our data indicated that liarzozole was capable of directly inhibiting 24-hydroxylase activity, the enzyme involved in the first step of 1,25-(OH)₂D₃ inactivation, we next investigated the effect of liarzozole on the 1,25-(OH)₂D₃ half-life. We treated two group of cells at various times, one with the single addition of 10 nM 1,25-(OH)₂D₃ and the other with a combination of 10 nM 1,25-(OH)₂D₃ and 1 μ M liarzozole. As anticipated, in cultures treated with 1,25-(OH)₂D₃ alone, the half-life of 1,25-(OH)₂D₃ was shorter (\sim 10 h) compared with that of cells treated with the combination (\sim 30 h; Fig. 4). Therefore, these data are consistent with the observation that liarzozole directly inhibits 24-hydroxylase activity, thereby prolonging 1,25-(OH)₂D₃ half-life. This finding provides one possible mechanism for the synergistic growth inhibitory effect of combination therapy with 1,25-(OH)₂D₃ and liarzozole.

Effect of 1,25-(OH)₂D₃ and liarzozole on VDR protein level

As previously shown in other cell culture systems, 1,25-(OH)₂D₃ and other vitamin D analogs induce homologous

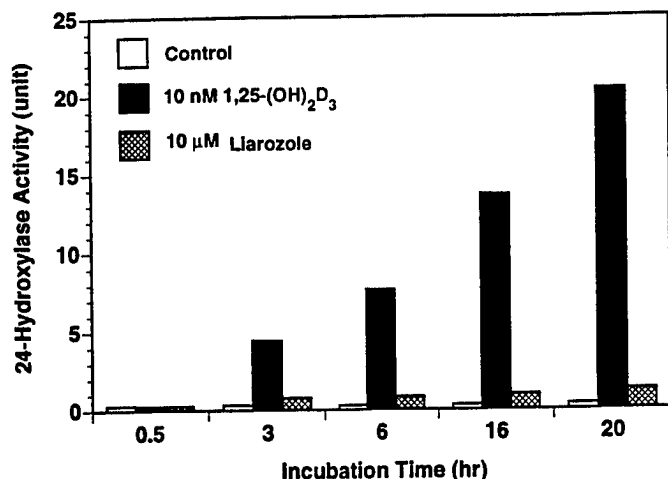


FIG. 2. Time course of the effect of 1,25-(OH)₂D₃ or liarazole on 24-hydroxylase activity in DU 145 cells. Cells were treated with 10 nM 1,25-(OH)₂D₃, 10 μM liarazole, or ethanol vehicle, and enzyme activity was measured at 0.5, 3, 6, 16, and 20 h. At 20 h, 10 nM 1,25-(OH)₂D₃ induced a 27-fold rise in 24-hydroxylase activity compared with the effect of vehicle. This is a representative experiment that was performed twice with similar results.

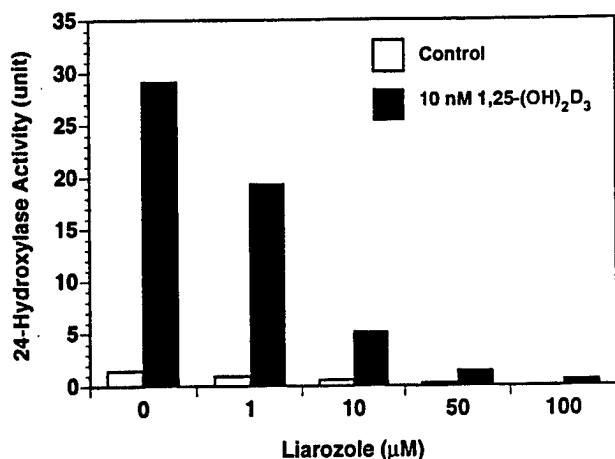


FIG. 3. Dose-dependent effect of liarazole on 24-hydroxylase activity in DU 145 cells. Cells were treated with 10 nM 1,25-(OH)₂D₃ for 20 h. Treated cells were subsequently incubated with liarazole at various concentrations (0, 1, 10, 50, and 100 μM) for 30 min before enzyme activity was measured. This is a representative experiment that was performed three times with similar results.

up-regulation of the VDR (21, 22). We next investigated whether liarazole, by inhibiting 24-hydroxylase activity and prolonging 1,25-(OH)₂D₃ half-life, is also capable of augmenting VDR up-regulation. Cells were treated with various concentrations of 1,25-(OH)₂D₃ (0.1, 1, and 10 nM) with and without the addition of 10 μM liarazole over a time course of 4 days. Protein extracts were made from these treated cells and were subjected to Western blot analysis to evaluate VDR content. Using the monoclonal antibody 9A7, the 50-kDa VDR protein was visualized. As shown in Fig. 5A, 1,25-(OH)₂D₃ alone resulted in a slight up-regulation of the VDR protein level (2-fold). Liarazole alone had no significant effect on VDR abundance. However, the combination of 1,25-(OH)₂D₃ and liarazole resulted in a 1,25-(OH)₂D₃ dose-dependent increase in the VDR protein level. This effect was

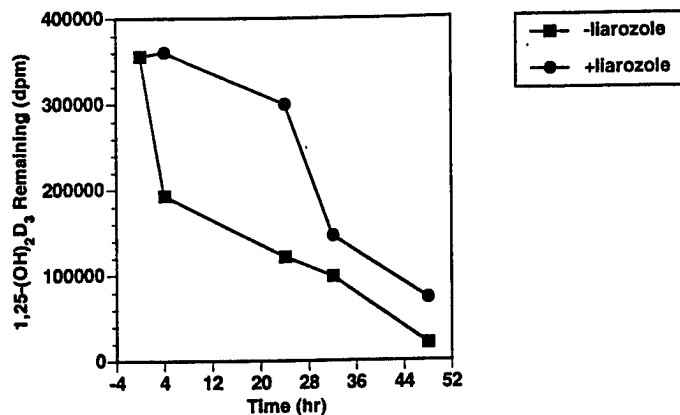


FIG. 4. Effect of liarazole on 1,25-(OH)₂D₃ half-life. DU 145 cells were incubated with [³H]1,25-(OH)₂D₃ (0.5 nM) plus unlabeled 1,25-(OH)₂D₃ (10 nM) in the absence or presence of 10 μM liarazole. Conditioned media were collected at various time points (0, 4, 24, 32, and 48 h), and the residual amount of unmetabolized [³H]1,25-(OH)₂D₃ was determined by TLC. This is a representative experiment performed twice with similar results.

observed most profoundly after a combination treatment with 10 nM 1,25-(OH)₂D₃ and 10 μM liarazole. This combination, as shown in Fig. 5B, resulted in a 5-fold increase in VDR abundance. Our data suggest that an increase in the VDR protein level may serve as a second and related mechanism, added to the prolonged 1,25-(OH)₂D₃ half-life, that contributes to the synergistic effect of 1,25-(OH)₂D₃ and liarazole inhibition of DU 145 cell growth.

Discussion

Our study was designed to investigate the combination of 1,25-(OH)₂D₃ and liarazole as a possible treatment for prostate cancer. We carried out our studies using an aggressive human prostate cancer cell line, DU 145, because of its resistance to the growth inhibitory effects of 1,25-(OH)₂D₃. We successfully inhibited DU 145 cell growth by 60% using the combination treatment of 1,25-(OH)₂D₃ and liarazole. In addition, we gained insight into the mechanism of the DU 145 cell unresponsiveness and the possibility of reversing the resistance with combination therapy.

As discussed earlier, at concentrations that are nontoxic, neither liarazole nor 1,25-(OH)₂D₃ alone substantially inhibited cell growth. Yet the combination of 1 μM liarazole and 10 nM 1,25-(OH)₂D₃ resulted in significant synergistic antiproliferative effects. Furthermore, this synergy is observed at a pharmacologically relevant concentration for both compounds. Based on this observation, we explored the possible mechanisms behind this synergy. We discovered that liarazole directly inhibited 24-hydroxylase activity in addition to inhibiting the already known P-450 enzymes, such as 4-hydroxylase and aromatase (15, 17, 18). As 24-hydroxylase is the initial enzyme for inactivating 1,25-(OH)₂D₃, we measured 1,25-(OH)₂D₃ half-life, and indeed, it was prolonged from 11 to 31 h. Therefore, by preventing rapid inactivation of 1,25-(OH)₂D₃ and prolonging the exposure time of cells to active hormone, DU 145 cells were able to respond to its antiproliferative effect. This provided the first possible mechanism for the synergistic activity of the 1,25-(OH)₂D₃/liarazole combination.

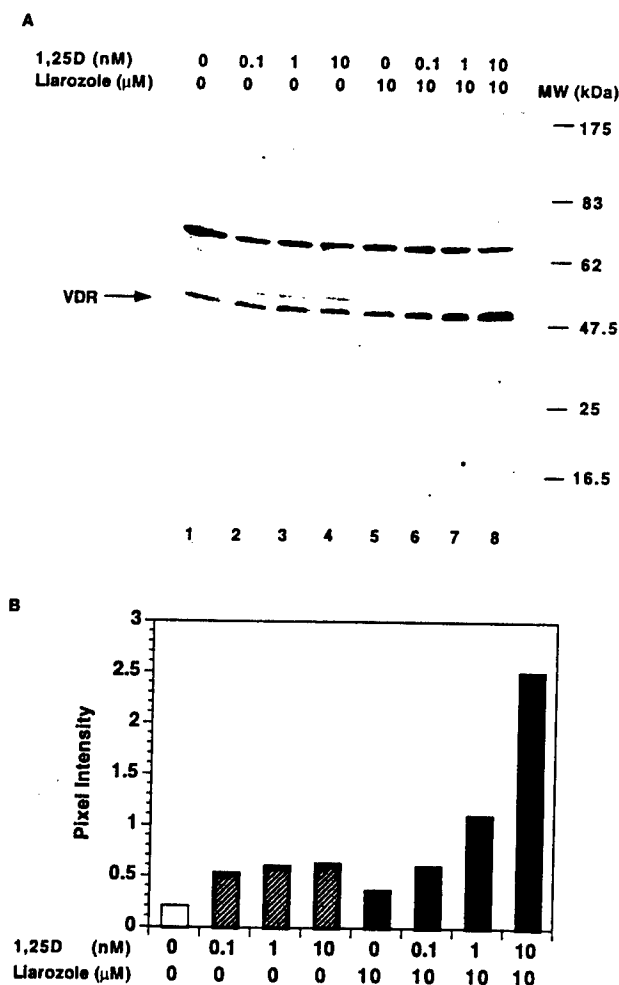


FIG. 5. A, Western blot analysis of VDR levels in DU 145 cells in response to various treatments. Cells were treated with ethanol or increasing concentrations of 1,25-(OH)₂D₃, liarazole, or 1,25-(OH)₂D₃ and liarazole for 4 days. High salt extracts were prepared, and 100 μg protein were loaded onto an 8% SDS-PAGE. After gel transfer, the blot was probed with anti-VDR monoclonal antibody 9A7, and the signal was detected using the enhanced chemiluminescence method. B, Densitometric analysis of Western blot. The pixel intensities of VDR bands were quantitated using a laser densitometer.

It is known that receptor regulation is an important mechanism for modulating target cell responsiveness to hormone (20, 23, 24). We explored the possibility that homologous up-regulation of the VDR would be enhanced in the presence of liarazole. Western blot analysis demonstrated a 5-fold increase in VDR protein level when cells were treated with 10 nM 1,25-(OH)₂D₃ and 10 μM liarazole compared with that after treatment with 1,25-(OH)₂D₃ alone. DU 145 cells treated with 1,25-(OH)₂D₃ alone only demonstrated a slight up-regulation of the VDR protein level. The possible explanation for this observation is that 1,25-(OH)₂D₃ is a potent stimulus of 24-hydroxylase activity; therefore, this would induce rapid degradation of 1,25-(OH)₂D₃, causing only a transient homologous up-regulation of VDR in DU 145 cells. Augmentation of VDR up-regulation has previously been reported using ketoconazole to inhibit 24-hydroxylase in a similar manner (25, 26). Enhanced VDR up-regulation is the second contributing mechanism explaining the liarazole synergistic interaction with 1,25-(OH)₂D₃. The increase in both ligand

and receptor is a plausible mechanism for the enhanced antiproliferative activity of the 1,25-(OH)₂D₃/liarazole combination therapy in DU 145 cells. It is of interest that analogs of 1,25-(OH)₂D₃ designed to prevent 24-hydroxylation, such as 19-nor-25,26-hexafluoro-1,25-(OH)₂D₃, inhibit DU145 cell proliferation (9).

Our data suggest that DU 145 cells are more responsive to the antiproliferative effect of 1,25-(OH)₂D₃ when both its hormone and receptor are increased. Although the presence of VDR is essential for 1,25-(OH)₂D₃ activity (27), the level of VDR abundance in different prostate cancer cell lines by itself is not necessarily predictive of the amplitude of hormonal response (5, 28). However, in a given cell, increased abundance of receptor does appear to predict the extent of hormonal responsiveness, and increased or decreased receptor levels are usually correlated with increased and decreased responsiveness, respectively (20, 23, 24).

It should be noted that Zhao *et al.* have shown that combination therapy with either ketoconazole or liarazole and 1,25-(OH)₂D₃ or its analogs is cell type specific (13). That finding supports the concept that differences in cellular metabolism can at least partially explain the different potencies of various vitamin D analogs and differences in antiproliferative activity between different cancer cells. The fact that some cells are substantially growth inhibited by 1,25-(OH)₂D₃ alone (LNCaP and primary cultures) and other cells are not (DU 145) depends on a combination of factors, including, but not limited to, VDR abundance and inducible 24-hydroxylase activity (5, 7, 28). Liarazole in combination with 1,25-(OH)₂D₃ improves both parameters; by increasing VDR abundance and inhibiting 24-hydroxylase activity, it allows the otherwise resistant DU 145 cell to be growth arrested by 1,25-(OH)₂D₃. In preliminary experiments, liarazole also augmented the growth inhibitory activity of 1,25-(OH)₂D₃ in PC-3 and LNCaP cells, but to a much lesser extent (data not shown) than shown here for DU145 cells. The smaller augmentation was probably due to the greater antiproliferative activity of 1,25-(OH)₂D₃ alone in these cells (5) as well as the lesser induction of 24-hydroxylase in these cell lines (7), making the liarazole action to inhibit 24-hydroxylase less essential for 1,25-(OH)₂D₃-mediated growth inhibition.

Another possible mechanism for the enhanced antiproliferative effect in the presence of liarazole is its ability to inhibit retinoid metabolism, leading to increased retinoid levels (15, 18, 29). Retinoids have been shown to inhibit various cancer cell lines, including prostate cancer (30), and to be synergistic with 1,25-(OH)₂D₃ in inhibiting prostate cancer cell growth (10, 31). In fact, the beneficial effect of liarazole in patients with prostate cancer is attributed to this activity (15, 18, 29). We investigated the possibility that this activity might be contributing to the growth inhibition in our experiments. We treated DU 145 cells with a combination of 1,25-(OH)₂D₃ and increasing concentrations of retinoic acid to mimic the liarazole effect. We observed only a slight enhancement of growth inhibition (data not shown). As liarazole alone had no antiproliferative activity, and retinoids were not added in our standard combination experiments, we believe that the inhibition of retinoid metabolism does not substantially contribute to the effects that we have seen in cultured cells.

However, in patients, the ability of liarazole to inhibit retinoid metabolism would be expected to further enhance the synergistic activity that we have demonstrated.

The mechanism(s) by which 1,25-(OH)₂D₃ inhibits the growth of prostate cancer cells is complex, multifactorial, and different in different cell lines. Several investigators have reported that treatment with 1,25-(OH)₂D₃ causes LNCaP cells to accumulate in the G₁ phase of the cell cycle (10, 11). 1,25-(OH)₂D₃ also elicits a reduction of cyclin-dependent kinase 2 activity and an increase in the level of hypophosphorylated retinoblastoma (Rb) protein, which is a critical regulator of the G₁/S checkpoint (11). Interestingly, DU 145 cells lack functional Rb protein. However, ectopic expression of functional Rb in DU 145 cells was not sufficient to restore the growth response to 1,25-(OH)₂D₃ (32). Others have found that growth inhibition of prostate cancer cells by a potent vitamin D analog involves the induction of p21^{waf1}, p27^{kip1}, and E-cadherin (9). In addition, we have demonstrated that 1,25-(OH)₂D₃ significantly regulates androgen receptor gene expression, which contributes to the regulation of LNCaP cell growth (33). Therefore, the mechanism by which 1,25-(OH)₂D₃ inhibits cell proliferation involves multiple signaling pathways and differs in various prostate cancer cell lines.

In summary, our data suggest that liarazole directly inhibits 24-hydroxylase activity, thereby effectively prolonging the 1,25-(OH)₂D₃ half-life. The increase in the 1,25-(OH)₂D₃ half-life resulted in enhanced up-regulation of VDR protein levels. We believe that this combination of increased 1,25-(OH)₂D₃ hormone levels as well as augmented VDR abundance represents the principal mechanism for the synergistic effect of 1,25-(OH)₂D₃ and liarazole in our experiments. However, additional mechanisms may play a role in the synergistic effect of this combination in DU 145 cells. In conclusion, the novel combination of liarazole and 1,25-(OH)₂D₃ therapy may serve as an effective treatment regimen for prostate cancer patients.

Acknowledgments

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References

- Feldman D, Malloy PJ, Gross C 1996 Vitamin D: metabolism and action. In: Marcus R, Feldman D, Kelsey J (eds) Osteoporosis. Academic Press, New York, pp 205-235
- Pols HAP, Birkenhager JC, Foekens JA, Van Leeuwen JPTM 1990 Vitamin D: a modulator of cell proliferation and differentiation. *J Steroid Biochem* 37:873-876
- Walters MR 1992 Newly identified actions of the vitamin D endocrine system. *Endocr Rev* 13:719-764
- Bikle DD 1992 Clinical counterpoint: vitamin D: new actions, new analogs, new therapeutic potential. *Endocr Rev* 13:765-784
- Skowronski RJ, Peehl DM, Feldman D 1993 Vitamin D and prostate cancer: 1,25-dihydroxyvitamin D₃ receptors and actions in human prostate cancer cell lines. *Endocrinology* 132:1952-1960
- Schwartz GG, Oeler TA, Uskokovic MR, Bahnson RR 1994 Human prostate cancer cells: inhibition of proliferation by vitamin D analogs. *Anticancer Res* 14:1077-1081
- Miller GJ, Stapleton GE, Hedlund TE, Moffatt KA 1995 Vitamin D receptor expression, 24-hydroxylase activity, and inhibition of growth by 1 α ,25-dihydroxyvitamin D₃ in seven human prostatic carcinoma cell lines. *Clin Cancer Res* 1:997-1003
- Skowronski RJ, Peehl DM, Feldman D 1995 Actions of vitamin D₃ analogs on human prostate cancer cell lines: comparison with 1,25-dihydroxyvitamin D₃. *Endocrinology* 136:20-26
- Campbell MJ, Elstner E, Holden S, Uskokovic M, Koeffler HP 1997 Inhibition of proliferation of prostate cancer cells by a 19-nor-hexafluoride vitamin D₃ analogue involves the induction of p21^{waf1}, p27^{kip1} and E-cadherin. *J Mol Endocrinol* 19:15-27
- Blutt SE, Allegretto EA, Pike JW, Weigel NL 1997 1,25-Dihydroxyvitamin D₃ and 9-*cis*-retinoic acid act synergistically to inhibit the growth of LNCaP prostate cells and cause accumulation of cells in G₁. *Endocrinology* 138:1491-1497
- Zhuang SH, Burnstein KL 1998 Antiproliferative effect of 1 α ,25-dihydroxyvitamin D₃ in human prostate cancer cell line LNCaP involves reduction of cyclin-dependent kinase 2 activity and persistent G₁ accumulation. *Endocrinology* 139:1197-1207
- Peehl DM, Skowronski RJ, Leung GK, Wong ST, Stamey TA, Feldman D 1994 Antiproliferative effects of 1,25-dihydroxyvitamin D₃ on primary cultures of human prostatic cells. *Cancer Res* 54:805-810
- Zhao J, Tan BK, Marcelis S, Verstuyf A, Bouillon R 1996 Enhancement of antiproliferative activity of 1 α ,25-dihydroxyvitamin D₃ (analogues) by cytochrome P450 enzyme inhibitors is compound- and cell-type specific. *J Steroid Biochem Mol Biol* 57:197-202
- Feldman D 1986 Ketoconazole and other imidazole derivatives as inhibitors of steroidogenesis. *Endocr Rev* 7:409-420
- De Coster R, Wouters W, Van Ginckel R, End D, Krekels M, Coene M-C, Bowden C 1992 Experimental studies with liarazole (R 75 251): an antitumoral agent which inhibits retinoic acid breakdown. *J Steroid Biochem Mol Biol* 43:197-201
- Seidmon EJ, Trump DL, Kreis W, Hall SW, Kurman MR, Ouyang P, Wu J, Kremer AB 1995 Phase I/II dose escalation study of liarazole in patients with stage D, hormone refractory carcinoma of the prostate. *Ann Surg Oncol* 2:550-556
- Bruynseels J, De Coster R, Van Rooy P, Wouters W, Coene M-C, Snoeck E, Raeymaekers A, Freyne E, Sanz G, Vanden Bussche G, Vanden Bossche H, Willemsens G, Janssen PAJ 1990 R 75251, a new inhibitor of steroid biosynthesis. *Prostate* 16:345-357
- Van Wauwe J, Van Nyen G, Coene M-C, Stoppie P, Cools W, Goossens J, Borghgraef P, Janssen PAJ 1992 Liarazole, an inhibitor of retinoic acid metabolism, exerts retinoid-mimetic effects *in vivo*. *J Pharmacol Exp Ther* 261:773-779
- Chen TL, Hauschka PV, Cabrales S, Feldman D 1986 The effects of 1,25-dihydroxyvitamin D₃ and dexamethasone on rat osteoblast-like primary cell cultures: receptor occupancy and functional expression patterns for three different bioresponses. *Endocrinology* 118:250-259
- Krishnan AV, Feldman D 1992 Cyclic adenosine 3',5'-monophosphate up-regulates 1,25-dihydroxyvitamin D₃ receptor gene expression and enhances hormone action. *Mol Endocrinol* 6:198-206
- Costa EM, Hirst MA, Feldman D 1985 Regulation of 1,25-dihydroxyvitamin D₃ receptors by vitamin D analogs in cultured mammalian cells. *Endocrinology* 117:2203-2210
- Costa E, Feldman D 1987 Measurement of 1,25-dihydroxyvitamin D₃ receptor turnover by dense amino acid labeling: changes during up-regulation by vitamin D metabolites. *Endocrinology* 120:1173-1178
- Chen TL, Li JM, VanYe T, Cone CM, Feldman D 1986 Hormonal responses to 1,25-dihydroxyvitamin D₃ in cultured mouse osteoblast-like cells: modulation by changes in receptor level. *J Cell Physiol* 126:21-28
- Krishnan A, Feldman D 1997 Regulation of vitamin D receptor abundance. In: Feldman D, Glorieux FH, Pike JW (eds) Vitamin D. Academic Press, San Diego, pp 179-200
- Pols HA, Schilte HP, Visser TJ, Birkenhager JC 1987 Effect of ketoconazole on metabolism and binding of 1,25-dihydroxyvitamin D₃ by intact rat osteogenic sarcoma cells. *Biochim Biophys Acta* 931:115-119
- Reinhardt TA, Horst RL 1989 Ketoconazole inhibits self-induced metabolism of 1,25-dihydroxyvitamin D₃ and amplifies 1,25-dihydroxyvitamin D₃ receptor up-regulation in rat osteosarcoma cells. *Arch Biochem Biophys* 272:459-465
- Hedlund TE, Moffatt KA, Miller GJ 1996 Vitamin D receptor expression is required for growth modulation by 1 α ,25-dihydroxyvitamin D₃ in the human prostatic carcinoma cell line ALVA-31. *J Steroid Biochem Mol Biol* 58:277-288
- Zhuang SH, Schwartz GG, Cameron D, Burnstein KL 1997 Vitamin D receptor content and transcriptional activity do not fully predict antiproliferative effects of vitamin D in human prostate cancer cell lines. *Mol Cell Endocrinol* 126:83-90
- Miller VA, Rigas JR, Muindi JRF, Tong WP, Venkatraman E, Kris MG, Warrell RP 1994 Modulation of all-*trans* retinoic acid pharmacokinetics by liarazole. *Cancer Chemother Pharmacol* 34:522-526
- Fong C, Sutkowski DM, Braun EJ, Bauer KD, Sherwood ER, Lee C, Kozlowski JM 1993 Effect of retinoic acid on the proliferation and secretory activity of androgen-responsive prostatic carcinoma cells. *J Urol* 149:1190-1194
- Peehl DM, Wong ST, Cramer SD, Gross C, Feldman D 1995 Suramin, hydrocortisone and retinoic acid modify inhibitory effects of 1,25-dihydroxyvitamin D₃ on prostatic epithelial cells. *Urol Oncol* 1:188-194
- Gross C, Skowronski RJ, Plymate SR, Rhim JS, Peehl DM, Feldman D 1996 Simian virus 40-, but not human papillomavirus-, transformation of prostatic epithelial cells results in loss of growth-inhibition by 1,25-dihydroxyvitamin D₃. *Int J Oncol* 8:41-47
- Zhao XY, Ly LH, Peehl DM, Feldman D 1997 1 α ,25-Dihydroxyvitamin D₃ actions in LNCaP human prostate cancer cells are androgen-dependent. *Endocrinology* 138:3290-3298

Induction of Androgen Receptor by 1 α ,25-Dihydroxyvitamin D₃ and 9-cis Retinoic Acid in LNCaP Human Prostate Cancer Cells*

XIAO-YAN ZHAO, LAN H. LY, DONNA M. PEEHL, AND DAVID FELDMAN

Departments of Medicine (X.-Y.Z., L.H.L., D.F.) and Urology (D.M.P.), Stanford University School of Medicine, Stanford, California 94305

ABSTRACT

We have recently shown that 1 α ,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃] inhibits proliferation of LNCaP cells, an androgen-responsive human prostate cancer cell line. Also, 1,25-(OH)₂D₃ increases androgen receptor (AR) abundance and enhances cellular responses to androgen in these cells. In the current study, we have investigated the mechanism by which 1,25-(OH)₂D₃ regulates AR gene expression and the involvement of AR in the 1,25-(OH)₂D₃- and 9-cis retinoic acid (RA)-mediated growth inhibition of LNCaP cells. Northern blot analyses demonstrated that the steady-state messenger RNA (mRNA) level of AR was significantly increased by 1,25-(OH)₂D₃ in a dose-dependent manner. Time-course experiments revealed that the increase of AR mRNA by 1,25-(OH)₂D₃ exhibited delayed kinetics. In response to 1,25-(OH)₂D₃, AR mRNA levels were first detected to rise at 8 h and reached a maximal induction of 10-fold over the untreated control at 48 h; the effect was sustained at 72 h. Furthermore, the induction of AR mRNA by 1,25-(OH)₂D₃ was completely abolished by incubation of cells with cycloheximide, a protein synthesis inhibitor.

1,25-(OH)₂D₃ was unable to induce expression of an AR promoter-luciferase reporter. Together, these findings indicate that the stimulatory effect of 1,25-(OH)₂D₃ on AR gene expression is indirect. Western blot analyses showed an increase of AR protein in 1,25-(OH)₂D₃-treated cells. This increased expression of AR was followed by 1,25-(OH)₂D₃-induced inhibition of growth in LNCaP cells. Similar to 1,25-(OH)₂D₃, 9-cis RA also induced AR mRNA expression, and the effect of both hormones was additive. Moreover, 1,25-(OH)₂D₃ and 9-cis RA acted synergistically to inhibit LNCaP cell growth. These antiproliferative effects of 1,25-(OH)₂D₃ and 9-cis RA, alone or in combination, were blocked by the pure AR antagonist, Casodex. In conclusion, our results demonstrate that growth inhibition of LNCaP cells by 1,25-(OH)₂D₃ and 9-cis RA is mediated by an AR-dependent mechanism and preceded by the induction of AR gene expression. This finding, that differentiating agents such as vitamin D and A derivatives are potent inducers of AR, may have clinical implications in the treatment of prostate cancer. (*Endocrinology* 140: 1205–1212, 1999)

1 α ,25-DIHYDROXYVITAMIN D₃ [1,25-(OH)₂D₃], the active metabolite of vitamin D, regulates calcium homeostasis in the body by actions in the intestine, bone, kidney, and parathyroid glands (1, 2). Recently, 1,25-(OH)₂D₃ has also been shown to have nonclassical actions. For example, the hormone exerts antiproliferative and prodifferentiating effects on many cell types, including cells derived from myeloid, breast, colon, and prostate tissues (3–6). Biologic responses of target cells to 1,25-(OH)₂D₃ are mediated by its nuclear receptor, the vitamin D receptor (VDR) (7). The VDR belongs to the steroid/thyroid/retinoid receptor superfamily (1, 2). Numerous studies indicate that VDR controls target gene transcription by forming a heterodimeric complex with the retinoid X receptor (RXR), the receptor for 9-cis retinoic acid (RA), and binding to the vitamin D response element (VDRE) present in the promoter region of target genes.

Our group (8, 9), as well as others (10), have shown that VDRs are present in established human prostate cancer cell lines, as well as primary cultures of normal prostate and

cancer cells (11). Moreover, 1,25-(OH)₂D₃ and its analogs significantly inhibit cellular proliferation of prostate cancer cells, including LNCaP (8, 9, 12–20). LNCaP cells express both the VDR and the androgen receptor (AR). Our recent studies (21) and those of others (15, 22) have demonstrated that cross-talk between 1,25-(OH)₂D₃ and androgens exists and that the antiproliferative actions of 1,25-(OH)₂D₃ in LNCaP cells are androgen-dependent. Blutt *et al.* (17) have shown that 9-cis RA acts synergistically with 1,25-(OH)₂D₃ to inhibit LNCaP cell growth.

Because cellular responsiveness to androgen depends on AR abundance, in the present study, we have analyzed the ability of 1,25-(OH)₂D₃ and 9-cis RA to regulate the level of AR gene expression in these cells. We found that 1,25-(OH)₂D₃ increased the levels of AR messenger RNA (mRNA) and AR protein in a concentration- and time-dependent manner. Such regulatory effects of 1,25-(OH)₂D₃ on AR gene expression required *de novo* protein synthesis. Furthermore, the stimulatory effect of 1,25-(OH)₂D₃ on AR mRNA was also enhanced by 9-cis RA. Because it has been reported that the antiproliferative effects of 1,25-(OH)₂D₃ on LNCaP cells can be synergistically enhanced by the addition of 9-cis RA (17), we examined the involvement of AR in the antiproliferative action of 9-cis RA, as well as 1,25-(OH)₂D₃. Using the pure AR antagonist, Casodex, we demonstrated that AR blockade prevented the growth inhibitory activity of both 1,25-(OH)₂D₃ and 9-cis RA. In contrast, Casodex did not affect the antiproliferative activity of dibutyryl cAMP, a well-known

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Address all correspondence and requests for reprints to: David Feldman, M.D., Division of Endocrinology, Stanford University Medical Center, Room S-005, Stanford, California 94305-5103.

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up-regulator of AR in LNCaP cells (23). Our studies demonstrate that both 1,25-(OH)₂D₃ and 9-cis RA up-regulate AR mRNA levels in LNCaP cells and that growth inhibition mediated by 1,25-(OH)₂D₃ and 9-cis RA requires the action of AR.

Materials and Methods

Materials

1,25-(OH)₂D₃ was the generous gift of Dr. M. Uskokovic (Hoffmann-La Roche, Inc., Nutley, NJ). Bicalutamide (Casodex or ICI 17,334) was a gift from Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK). Aprepitin, pepstatin, and soybean trypsin inhibitor were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Tissue culture media were purchased from Mediatech (Herndon, VA). All other reagents, except where indicated, were purchased from Sigma Chemical Co. (St. Louis, MO). The anti-AR monoclonal antibody F39.4 and the human AR complementary DNA (cDNA) were generous gifts from Dr. TH Van der Kwast (Erasmus University, Rotterdam, Netherlands) and Dr. M. McPhaul (University of Texas Southwestern Medical Center, Dallas, TX), respectively. FBS was obtained from Gibco BRL (Gaithersburg, MD). Charcoal-stripped FBS (CSS) was purchased from Sigma Chemical Co.

Cell culture and hormone treatment

The LNCaP human prostate carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were routinely cultured in RPMI-1640 medium supplemented with 5% FBS and antibiotics (FBS medium), at 37°C in a humidified atmosphere of 5% CO₂. For experiments, LNCaP cells were trypsinized and seeded at an appropriate density, and hormonal treatments were initiated, the next day, in FBS medium or in RPMI-1640 medium supplemented with 5% CSS and antibiotics (CSS medium).

Hormone stocks [1,25-(OH)₂D₃, 9-cis RA, and Casodex] were prepared in 100% ethanol at a concentration 1000-fold higher than the working concentrations. Fresh culture media were premixed with hormone stock and then added to triplicate wells. Media and hormone were replenished every 2 days. Hormone received ethanol vehicle at a concentration equal to that in controls.

Assay of cell proliferation

Cell proliferation was assessed by measurement of attained cell mass using an assay of DNA content. As previously described (21), LNCaP cells were seeded in six-well tissue culture plates (Becton Dickinson and Co., Lincoln Park, NJ), at a density of 50,000 cells per well, in 3 ml RPMI-1640 containing 5% FBS. After incubation for 24 h, the medium was replaced with fresh medium containing 5% FBS (FBS medium). Cells were treated with vehicle (ethanol, final concentration 0.1%), 1,25-(OH)₂D₃, 9-cis RA, dibutyl cAMP, or Casodex. On the sixth day, cell monolayers were processed for DNA assay using the method of Burstein (24). DNA content of each treatment was derived from the mean value of triplicate wells in an experiment. Each experiment was repeated three times.

Western blot analysis

Cells were treated with ethanol or 1,25-(OH)₂D₃ (10 nM) in RPMI-1640 medium containing 5% CSS (CSS medium) for 2 days. They were harvested, and sonicated extracts were prepared as described. Aliquots of 100 µg protein were heated in SDS sample buffer at 95°C, for 5 min, before electrophoresis in an 8% SDS-polyacrylamide gel. After electrophoresis, the gels were transferred and processed as previously described (25). After transfer, the blots were incubated with anti-AR monoclonal antibody F39.4 (1:100 dilution) for 1 h, at room temperature, with gentle shaking. The blots were washed and then incubated with a horseradish peroxidase-conjugated rabbit antimouse IgG (1:1000 dilution) for 1 h at room temperature. Blots were re-washed and developed with the Enhanced Chemiluminescence (ECL) System system, according to the manufacturer's instructions (Amersham Chemical Co.).

Steroid receptor ligand-binding assay

LNCaP cells were seeded at a density of 150,000 cells per 100-mm dish in 10-ml medium containing 5% FBS or 5% CSS. At the end of the 6-day incubation with hormone (at concentrations of 0, 1, and 10 nM), cell monolayers were harvested, and high-salt nuclear extracts were made as previously described (21). Protein concentration of the extract was determined (26). In a typical binding experiment, 200 µl soluble extract (1–2 mg protein/ml) were incubated with 10 nM concentration of [³H]-5α-dihydrotestosterone (DHT) for 16–20 h at 4°C. Bound and free hormone were separated by hydroxylapatite (21). Specific binding was calculated by subtracting nonspecific binding (obtained in the presence of a 250-fold excess of radioinert DHT) from the total binding (measured in the absence of radioinert steroid). Data were expressed as femtomoles [³H]-DHT bound per milligram protein.

Northern blot analysis

Northern blot analysis was performed as previously described (8, 11). Briefly, semiconfluent LNCaP cells were treated with graded concentrations of 1,25-(OH)₂D₃, or 5 mM dibutyl cAMP, or 9-cis RA in FBS medium and in CSS medium for 24 h before isolation of total RNA. Ten micrograms of total RNA were denatured, fractionated by electrophoresis, and transferred to Hybond-N nylon membrane (Amersham), as previously described (8, 11). The bound RNA was immobilized by UV cross-linking and then hybridized with a random primed [³²P]-labeled 0.8-kb *HindIII*-*BamHI* fragment of the human AR cDNA at 60°C. To control for RNA sample loading and transfer, Northern blots were also hybridized with a [³²P]-labeled 0.9-kb *EcoRI* fragment of the human cDNA for the ribosomal protein gene L7 (8, 11). The silver grain pixel intensity of each AR and L7 band was scanned by a densitometer, and the data were integrated by scanner software and indexed to the corresponding levels of L7 mRNA.

AR promoter-luciferase reporter gene assay

LNCaP cells were seeded at 3 × 10⁶ cells/dish in 60-mm tissue culture dishes (Corning, Inc., Corning, NY) in RPMI-1640 medium containing 5% FCS and antibiotics. A 6-kb promoter-luciferase reporter was transfected using a calcium-phosphate method (23). Each transfection contained 1 µg pAR-LUC DNA (Drs. G. Mora and D. Tindall, personal communication) and 0.1 µg pSV-Renilla DNA. The control plasmid pSV-Renilla was used to monitor transfection efficiency. Cells were harvested after 32 h of incubation with tested compounds at 37°C. Luciferase activity was employed to measure induction using Promega Corp. (Madison, WI) dual luciferase assay system on luminometer TD-20 (Turner Design, Sunnyvale, CA). The results were expressed as the ratio of luciferase activity to Renilla activity.

Statistical analysis

ANOVA was used to assess the statistical significance of the difference. *P* < 0.05 was considered significant.

Results

We have recently demonstrated that the antiproliferative action of 1,25-(OH)₂D₃ in LNCaP cells is androgen-dependent (21). Here, we investigate further the interaction between 1,25-(OH)₂D₃ and androgen signaling pathways by exploring the mechanism of 1,25-(OH)₂D₃ regulation of AR gene expression in these cells. We also examine the possible involvement of AR in the synergistic antiproliferative actions of 1,25-(OH)₂D₃ and 9-cis RA on LNCaP cells.

Dose response effect of 1,25-(OH)₂D₃ on AR mRNA

The effect of 1,25-(OH)₂D₃ on steady-state AR mRNA levels was assessed by Northern blot analysis. We have used two culture conditions (FBS medium and CSS me-

dium) in this set of experiments and have observed similar results. As shown in Fig. 1, LNCaP cells express a major transcript of AR at 11 kb. In Fig. 1A, the cells were treated in CSS medium for 24 h with increasing concentrations of $1,25-(\text{OH})_2\text{D}_3$ (0–100 nM), and AR mRNA transcripts increased in a dose-dependent manner. The increased AR mRNA levels became evident with a concentration of $1,25-(\text{OH})_2\text{D}_3$ at 1 nM (lane 3). Increasing the $1,25-(\text{OH})_2\text{D}_3$ concentration caused further induction of AR mRNA (lanes 4–5). The levels of AR mRNA were quantitatively determined by densitometric scanning of the autoradiographs, with correction for the L7 mRNA signal (Fig. 1B). At 100 nM of $1,25-(\text{OH})_2\text{D}_3$, more than 5-fold up-regulation of AR mRNA was detected (lane 5). When we carried out the experiment using FBS medium (Figs. 1, C and D), we also detected a significant up-regulation of AR mRNA in LNCaP cells in response to $1,25-(\text{OH})_2\text{D}_3$ treatment for

24 h. Hence, $1,25-(\text{OH})_2\text{D}_3$ increased AR mRNA expression in LNCaP cells in a dose-dependent manner in either CSS medium or FBS medium.

Time-course of AR mRNA expression in response to $1,25-(\text{OH})_2\text{D}_3$

In Fig. 2, time-course experiments using CSS medium revealed that addition of 10 nM $1,25-(\text{OH})_2\text{D}_3$ to LNCaP cells increased AR mRNA levels by 8 h. The AR mRNA levels peaked at 48 h in the treated cells, with a 10-fold higher level, compared with the untreated cells at the concurrent time point, and this inductive effect of $1,25-(\text{OH})_2\text{D}_3$ was sustained at 72 h. No change in AR mRNA could be detected at 4 h, suggesting a delayed primary response of AR gene expression to $1,25-(\text{OH})_2\text{D}_3$.

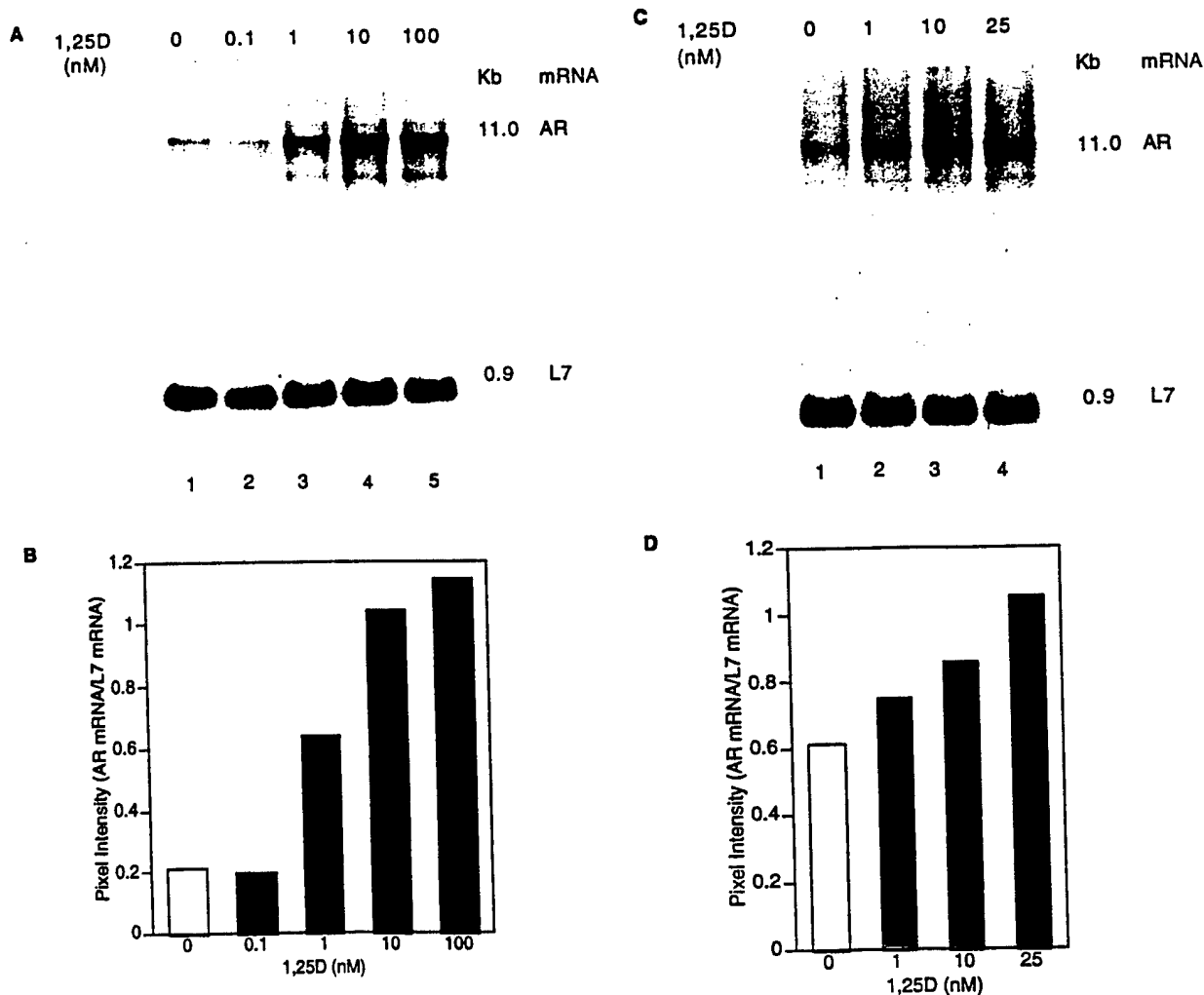


FIG. 1. Dose-dependent effect of $1,25-(\text{OH})_2\text{D}_3$ on AR mRNA levels in LNCaP cells. A, Northern blot analysis in CSS medium. LNCaP cells were treated with $1,25-(\text{OH})_2\text{D}_3$, at the indicated concentrations, for 24 h in RPMI medium containing 5% charcoal stripped serum. Total RNA was isolated, and the RNA blot was hybridized with a ^{32}P -labeled 712-bp *HindIII-EcoRI* fragment of the human AR cDNA at 60°C. The blot was simultaneously probed for expression of the L7 ribosomal protein gene as a control for sample loading and transfer. B, The pixel intensity of each AR band in panel A was scanned by computing densitometer, and the data were integrated by scanner software and indexed to the corresponding levels of L7 mRNA. C, Northern blot analysis in FBS medium. LNCaP cells were treated with $1,25-(\text{OH})_2\text{D}_3$, at the indicated concentrations, for 24 h in RPMI medium containing 5% FBS. Total RNA was isolated, and the RNA blot was hybridized with a ^{32}P -labeled human AR cDNA and the L7 gene. D, The pixel intensity of each AR band indexed to L7 in panel C.

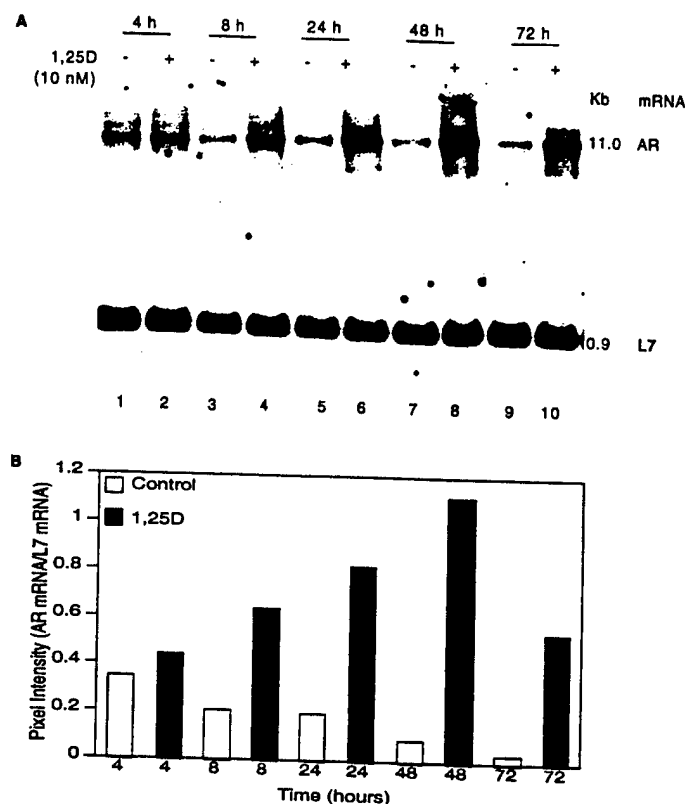


FIG. 2. Time-course of AR mRNA expression in LNCaP cells, in response to $1,25-(\text{OH})_2\text{D}_3$. A, Northern blot analysis. LNCaP cells were treated with $1,25-(\text{OH})_2\text{D}_3$, at 10 nM, for the indicated time period. Total RNA was isolated and analyzed by Northern blot using the human AR cDNA and L7 cDNA as probes. B, The pixel intensity of each AR band in panel A was scanned by computing densitometer, and the data were integrated by scanner software and indexed to the corresponding levels of L7 mRNA.

Up-regulation of the AR protein by $1,25-(\text{OH})_2\text{D}_3$

$1,25-(\text{OH})_2\text{D}_3$ also caused a concentration-dependent stimulation of AR protein expression in LNCaP cells cultured in CSS medium, as measured by Western blot analysis (Fig. 3). The major species of AR in LNCaP cells was detected as a single band, at 108 kDa, by monoclonal antibody F39.4. There was no detectable increase in AR protein levels after 24 h treatment (data not shown), but levels rose approximately 4-fold in cells treated with 10 nM $1,25-(\text{OH})_2\text{D}_3$ for 48 h. As seen for AR mRNA levels (Fig. 1), a detectable increase in AR protein was evident with 1 nM $1,25-(\text{OH})_2\text{D}_3$.

$[\text{H}]\text{DHT}$ -binding analyses demonstrated that $1,25-(\text{OH})_2\text{D}_3$ increased the AR content in LNCaP cells when they were cultured in either CSS medium or FBS medium. As we have reported (21), cells treated with 1 nM $1,25-(\text{OH})_2\text{D}_3$ in CSS medium showed a more than 2-fold increase in DHT-binding (from 197 ± 17.4 to 430 ± 9.6 fmol/mg protein, $n = 3$). Addition of 10 nM of $1,25-(\text{OH})_2\text{D}_3$ further up-regulated the AR content (from 197 ± 17.4 to 532 ± 60 fmol/mg, $n = 3$). Meanwhile, cells cultured in FBS medium exhibited a higher baseline DHT-binding than in CSS medium (378 ± 33.9 fmol/mg vs. 197 ± 17.4 fmol/mg, $n = 3$). $1,25-(\text{OH})_2\text{D}_3$ at 1 nM in FBS medium also increased the AR content (from 378 ± 33.9 to 451 ± 54 fmol/mg protein, $n = 3$). Therefore, $1,25-(\text{OH})_2\text{D}_3$ up-regulates the AR content in LNCaP cells in

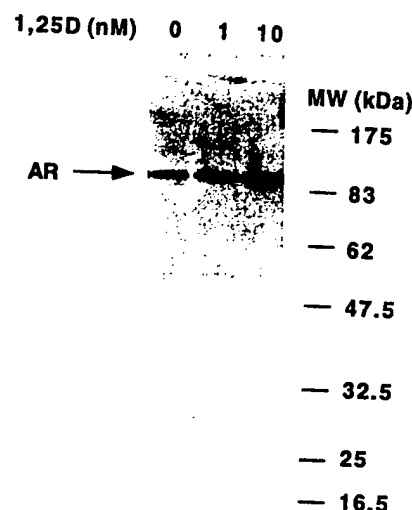


FIG. 3. Western blot analysis of AR protein in LNCaP cells. LNCaP cells were incubated, in RPMI medium containing 5% charcoal stripped serum, with the indicated dose of $1,25-(\text{OH})_2\text{D}_3$ for 2 days. High-salt protein extracts were electrophoresed in an 8% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose and probed with anti-AR monoclonal antibody F39.4. Immunoreactive bands were detected by incubation of blots with a secondary antibody (rabbit antimouse IgG), followed by ECL. Molecular weight standards are indicated. hAR is indicated by an arrow. The experiment was repeated twice, with similar results.

either growth condition. Interestingly, CSS medium allowed us to detect a clear up-regulation of AR because charcoal treatment removes endogenous steroids in serum that may interfere with $[\text{H}]\text{DHT}$ -binding.

Requirement of new protein synthesis for $1,25-(\text{OH})_2\text{D}_3$ regulation of AR

To determine whether $1,25-(\text{OH})_2\text{D}_3$ affected AR mRNA levels via a direct mechanism, LNCaP cells in CSS medium were treated for 24 h with $1,25-(\text{OH})_2\text{D}_3$ in the presence of the protein synthesis inhibitor cycloheximide (CHX) at various doses (0, 2, 5, and 10 $\mu\text{g}/\text{ml}$). As shown in Fig. 4, CHX blocked the $1,25-(\text{OH})_2\text{D}_3$ -induced increase in AR mRNA levels, such that in the presence of CHX and $1,25-(\text{OH})_2\text{D}_3$ (lane 3), AR mRNA levels were no higher than those in untreated cells (lane 1). The extent of blockade depended upon the concentration of CHX included in the media (lanes 3–5). Moreover, the effect of CHX could be detected at either 16 h (lane 6) or 24 h (lane 4).

In other studies, using a 6-kb AR promoter-luciferase reporter transfected into LNCaP cells, we attempted to directly induce expression of AR with $1,25-(\text{OH})_2\text{D}_3$. No increase in luciferase could be detected with $1,25-(\text{OH})_2\text{D}_3$, whereas dibutyl cAMP induced a 5-fold rise in luciferase (data not shown). Taken together, these findings indicate that $1,25-(\text{OH})_2\text{D}_3$ regulates AR mRNA expression via an indirect mechanism requiring new protein synthesis.

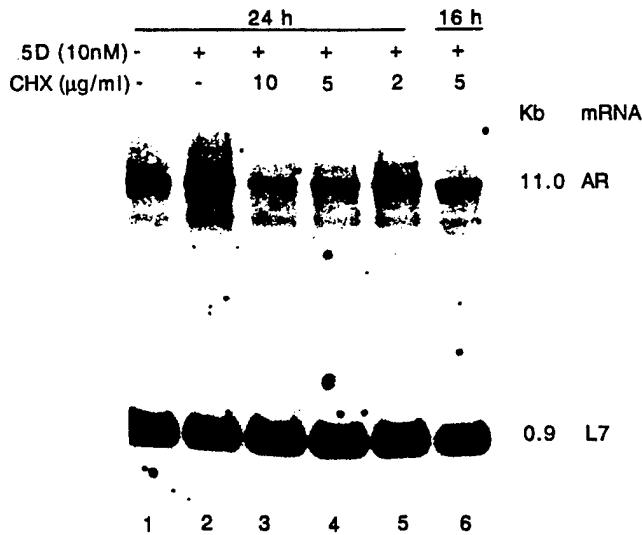


FIG. 4. Effect of CHX on the 1,25-(OH)₂D₃ induction of AR mRNA in LNCaP cells. Cells were treated with ethanol (lane 1) or 10 nM 1,25-(OH)₂D₃ (lanes 2-6), in the presence of CHX, at the indicated concentrations, for 24 h (lanes 1-5) or 16 h (lane 6). Total RNA was isolated and analyzed by Northern blot using the human AR cDNA and L7 cDNA as probes.

Enhancement of 1,25-(OH)₂D₃-mediated up-regulation of AR by 9-cis RA

It recently has been reported that 1,25-(OH)₂D₃ acts synergistically with 9-cis RA to inhibit LNCaP cell proliferation [7]. We therefore investigated the effect of 9-cis RA on 1,25-(OH)₂D₃ regulation of AR mRNA. Both culture conditions (FBS medium and CSS medium) gave similar results. As shown in Fig. 5, 1,25-(OH)₂D₃, at a dose of 10 nM, induced a 5-fold increase in AR mRNA levels (lane 1) over the control at 24 h (lane 2). LNCaP cells, treated with 100 nM 9-cis RA for 24 h, expressed 3-fold more AR mRNA (lane 3) than the untreated cells (lane 2). Combination treatment of 1,25-(OH)₂D₃ and 9-cis RA gave a more than 8-fold induction of AR mRNA (lane 4). Thus, although 1,25-(OH)₂D₃ was more effective than 9-cis RA in up-regulating AR mRNA, both hormones acted additively to increase AR gene expression in LNCaP cells.

Involvement of AR action in the antiproliferative response of 1,25-(OH)₂D₃ and 9-cis RA

We further tested the possible involvement of AR action in the antiproliferative effect of 1,25-(OH)₂D₃ and its synergism with 9-cis RA. As shown in Fig. 6A, 10 nM 1,25-(OH)₂D₃ inhibited LNCaP cell growth 50%, whereas 100 nM 9-cis RA only reduced proliferation by 10%. However, combination treatment with 1,25-(OH)₂D₃ and 9-cis RA caused 80% growth inhibition. Casodex was used to determine whether the 9-cis RA action was also AR-dependent. In the presence of Casodex, neither 1,25-(OH)₂D₃ nor 9-cis RA inhibited cell growth individually or in combination (Fig. 6A). On the other hand, dibutyryl cAMP, a known up-regulator of AR in LNCaP cells, inhibited cell proliferation in a dose-dependent manner (Fig. 6B). With maximal growth inhibition of 90% occurring at a dose of 5 mM dibutyryl cAMP, addition of Casodex, however, did not block this effect (Fig. 6B). These data imply

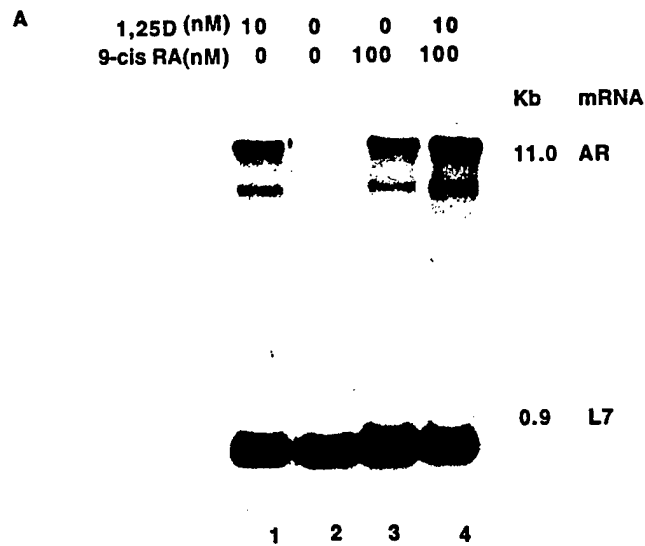


FIG. 5. Enhancement of 1,25-(OH)₂D₃ action by 9-cis RA in the induction of AR mRNA in LNCaP cells. A, Northern blot analysis. LNCaP cells were treated with 1,25-(OH)₂D₃ at 10 nM, or 9-cis RA at 100 nM, individually or in combination for 24 h. Total RNA was extracted and analyzed by Northern blot. B, The pixel intensity of each AR band in panel A was scanned by computing densitometer, and the data were integrated by scanner software and indexed to the corresponding levels of L7 mRNA.

that the actions of 1,25-(OH)₂D₃ and 9-cis RA on LNCaP cell growth are both AR-dependent. In contrast, cAMP, although an inducer of AR [23], inhibits LNCaP cell proliferation by an AR-independent mechanism.

Model of 1,25-(OH)₂D₃ and 9-cis RA action in LNCaP

The hormonal action of 1,25-(OH)₂D₃ is mediated by the VDR present in LNCaP cells. Figure 7 depicts the possible events in the 1,25-(OH)₂D₃ signaling pathway in these cells. 1,25-(OH)₂D₃ binds to the VDR and activates the receptor. The activated VDR controls target gene transcription by forming a heterodimer with the partner RXR and binding to the VDRE in the promoter region of a target gene. A 1,25-(OH)₂D₃ target gene (or genes) encodes protein(s) X, mediators of the up-regulation of AR mRNA in response to 1,25-(OH)₂D₃. The production of protein(s) X is CHX-sensitive. Both 1,25-(OH)₂D₃ and 9-cis RA, individually or in combi-

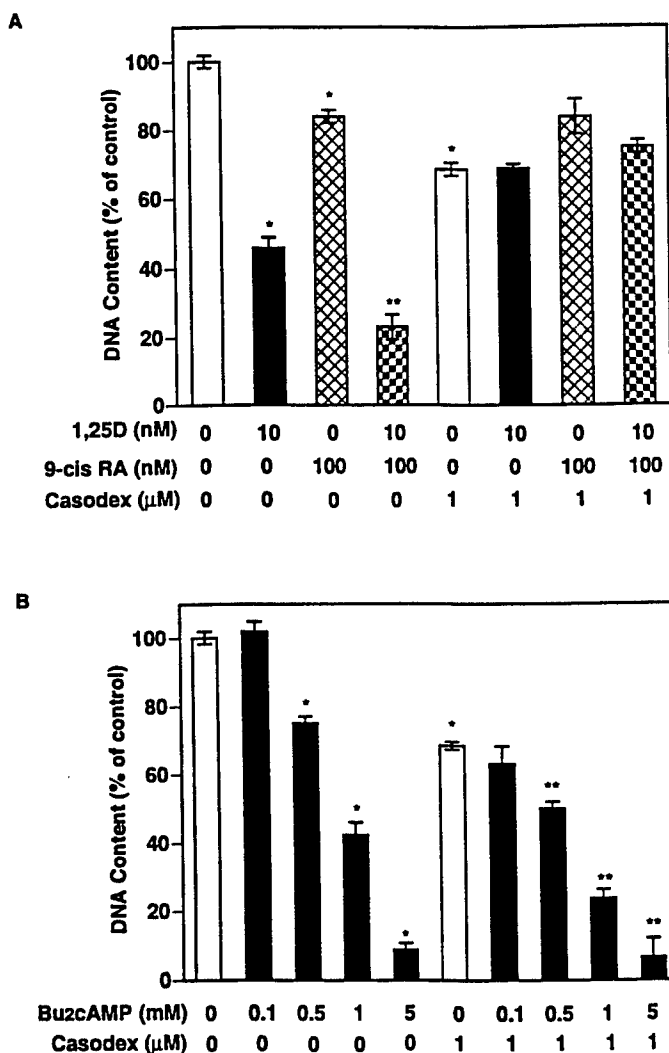


FIG. 6. Effect of Casodex on 1,25-(OH)₂D₃ and 9-cis RA-induced growth inhibition on LNCaP cells. A, LNCaP cells were treated with ethanol, 1,25-(OH)₂D₃ at 10 nM, or 9-cis RA at 100 nM, individually or both in the presence or absence of Casodex at 1 μM, for 6 days. Cellular DNA contents were determined by Burton's method. The data are expressed as percent of control, a mean of three triplicate samples ± SEM. *, *P* < 0.01, compared with the untreated control group; **, *P* < 0.05, compared with the single-treatment group. B, LNCaP cells were treated with dibutyl cAMP [Bu2cAMP], from 0–5 mM, in the presence or absence of Casodex at 1 μM, for 6 days.

nation, induce AR mRNA expression. The increased AR mRNA causes an increase in AR protein levels, which mediates the action of androgens on LNCaP cell growth. As we reported previously (23), either 1,25-(OH)₂D₃ or DHT was growth inhibitory on LNCaP when cells were cultured in FBS-containing medium. The androgen action is blocked by the AR antagonist, Casodex. Although both the AR mRNA and the AR protein are also induced in CSS medium, in the absence of androgens, 1,25-(OH)₂D₃ does not exhibit an antiproliferative effect on LNCaP cells in this culture system (21).

Discussion

The AR is the key element in the androgen signal transduction cascade, and it plays a critical role in the regulation

of growth and differentiation of the prostate. The data presented here demonstrate that 1,25-(OH)₂D₃ up-regulates AR gene expression at both mRNA and protein levels in LNCaP cells, an androgen-responsive human prostate cancer cell line. This inductive action of 1,25-(OH)₂D₃ was enhanced by 9-cis RA, which by itself also up-regulates AR expression in LNCaP cells. Our data show that growth inhibition induced by 1,25-(OH)₂D₃ alone or in combination with 9-cis RA was accompanied by increased AR expression. Moreover, the antiproliferative actions of 1,25-(OH)₂D₃ and 9-cis RA were AR-dependent and could be blocked by the AR antagonist, Casodex. Although androgens are not added in these experiments, androgens are present in the serum supplement to the culture medium (FBS medium), and we hypothesize that the amplitude of the androgen response is augmented by the increased levels of AR expressed in these cells after treatment with 1,25-(OH)₂D₃ and/or 9-cis RA. Support for the effect of androgens, in FBS medium, on cell growth was presented in our earlier studies (21). In the absence of androgens (for example, in CSS medium), 1,25-(OH)₂D₃ does not exhibit an antiproliferative action on LNCaP cells.

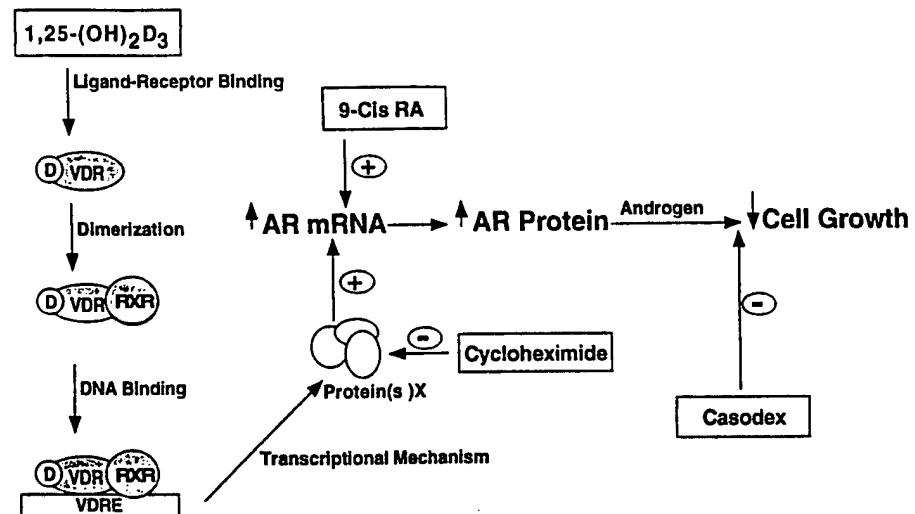
Nonetheless, our finding that androgen mediates the antiproliferative activity of 1,25-(OH)₂D₃ in LNCaP cells is not the situation in all prostate cancer cells. 1,25-(OH)₂D₃ inhibits the growth of AR-negative prostate cancer cell line PC-3, as well as primary cultures of human prostate cells. In contrast to LNCaP cells, mechanisms other than androgen signaling are responsible for the growth inhibitory effect of 1,25-(OH)₂D₃ on these cells.

It is of interest to consider whether increasing the abundance of a steroid receptor, such as the AR, will cause an increased amplitude of response, *i.e.* antiproliferation. Although it has been well demonstrated that the level of receptors in LNCaP does not necessarily predict the ligand potency of a hormonal response (8, 14, 19, 27), it is clear that the presence of a receptor is essential for a response (1, 2, 13, 14, 28). In a given cell, in the presence of a constant level of hormone, up-regulation of the receptor does cause an enhanced response, whereas down-regulation of the receptor diminishes the response (13, 14, 28–30). Therefore, we believe that up-regulation of AR, in these studies, is the mechanism of the enhanced antiproliferative effect.

The expression of the AR gene has been found to be induced by a number of agents in several systems, such as the rat ventral prostate (31), and in LNCaP human prostate cancer cells (32, 33). Growth factors such as FSH, EGF, and TGF-β regulate AR gene expression (34, 35). Activators of protein kinase A, such as forskolin and dibutyl cAMP, are the known up-regulators of AR in LNCaP cells (23). The induction of AR by these reagents was not detected in the two other commonly studied prostate cancer cell lines, PC-3 and DU 145, which do not express basal levels of AR mRNA (36).

1,25-(OH)₂D₃ is the most potent inducer of AR in LNCaP cells, among the three agents that we tested. Consistent with the reported data (23), we found that treatment of LNCaP cells with dibutyl cAMP for 24 h caused a 2-fold increase in AR mRNA levels (data not shown). In the same experiment, we observed an increase of 5-fold in AR mRNA, with 1,25-(OH)₂D₃ at 10 nM. 9-cis RA induced 3-fold induction of AR mRNA. It has been reported that dibutyl cAMP increases

FIG. 7. A tentative model of 1,25-(OH)₂D₃ and 9-cis RA action on LNCaP cells. Both 1,25-(OH)₂D₃ and 9-cis RA induce AR mRNA expression. The increased AR mRNA leads to an increase in AR protein levels. AR protein mediates androgen action in cell proliferation. The pure AR antagonist, Casodex, blocks AR action; in turn, it blocks the growth-inhibitory action of 1,25-(OH)₂D₃ and 9-cis RA.



AR gene transcription via the cAMP-response elements present in the 2.3-kb promoter region of the human AR gene (23). In contrast, the same promoter region of the AR gene seems to lack a VDRE and an RXRE. Computer searching of the 2.3-kb promoter region failed to identify a consensus sequence for these regulatory elements. Moreover, the luciferase reporter construct, driven by a 6-kb promoter region of the human AR gene, did not respond to 1,25-(OH)₂D₃ or 9-cis RA but did respond to dibutyl cAMP.

An indirect mechanism for 1,25-(OH)₂D₃ action to induce AR was supported by several findings taken together: the delayed time of AR mRNA rise in time-course experiments (Fig. 2), the CHX studies (Fig. 3), and the failure of the promoter to respond to 1,25-(OH)₂D₃ (data not shown). We refer to the indirect, CHX-inhibited mediator(s) of 1,25-(OH)₂D₃ action to up-regulate AR as protein(s) X. We surmise that, in the presence of CHX, 1,25-(OH)₂D₃ was unable to induce protein(s) X, and as a consequence, 1,25-(OH)₂D₃ failed to up-regulate AR mRNA (Fig. 4). It is interesting to speculate on the nature of protein(s) X. Protein(s) X may be related to the chaperon proteins, given the fact that several chaperons have been identified in the regulation of steroid receptors (37). Further studies are needed to elucidate this mechanism.

We did not detect AR up-regulation by 1,25-(OH)₂D₃ in two human breast cancer cells, either MCF-7 or T47D (unpublished data). Both MCF-7 and T47D cells express the VDR, as well as the AR. However, the levels of AR protein did not change in both cell lines when treated with 1,25-(OH)₂D₃. Therefore, induction of protein(s) X by 1,25-(OH)₂D₃ may be tissue-specific. At present, it is difficult to examine this point because of the limited number of human prostate cancer cells that exhibit the AR. We have, thus far, been unable to induce AR in cells that lack the AR, including primary cultures of prostate cancer cells and established cell lines PC-3 or DU 145. To determine whether AR induction by 1,25-(OH)₂D₃ is LNCaP cell-specific, we hope to study other AR-positive human prostate cancer cell lines as they become available.

The action of androgens to inhibit proliferation of cultured prostate cancer cells is an interesting finding. We and others

(21, 38) showed that LNCaP cells exhibit a biphasic growth response to DHT in charcoal-stripped serum-containing medium, with a growth stimulatory effect at a low concentration (less than 1 nM) and an inhibitory effect at a high concentration (greater than 1 nM). The levels of AR protein in LNCaP cells determine the concentration of DHT at which the stimulatory effect crosses over to an inhibitory effect. In other words, the stimulatory effect is favored at low abundance of AR, and an inhibitory effect at high abundance of AR (21). Liao and co-workers (39–41) found that high-passage LNCaP cells in an androgen-depleted medium express 10- to 20-fold higher AR levels and are growth inhibited by androgens *in vitro* and in an *in vivo* mouse model. Moreover, they demonstrated that G1 arrest of the high AR-expressing cells by androgen is caused by the induction of p27^{kip1}, which in turn inhibits Cdk2, a factor critical for cell cycle progression and proliferation (41). There are two additional examples to document the role of AR in the inhibition of growth of prostate cancer cells. Yuan *et al.* (42) have reported that PC-3 cells, stably transfected with the human AR cDNA, were growth inhibited by androgen. Recently, Zhau *et al.* (43) have established an androgen-repressed human prostate cancer cell line (ARCaP) derived from the ascites fluid of a patient with advanced metastatic disease, which is growth inhibited by androgens. Cumulatively, these findings support the hypothesis that higher levels of AR in cultured prostate cancer cells cause increased sensitivity to growth inhibition.

In summary, we have shown that the hormonally active forms of vitamin D and vitamin A are potent inducers of AR in LNCaP cells. Both 1,25-(OH)₂D₃ and 9-cis RA act in synergy to inhibit cell proliferation; moreover, their anti-proliferative actions can be blocked by the AR antagonist, Casodex. In conclusion, our study provides direct evidence for an important role of the AR in mediating the growth inhibitory actions of 1,25-(OH)₂D₃ and 9-cis RA in LNCaP cells. More importantly, the newly discovered AR-inducing property of both vitamins A and D suggests a possible application of these potential chemo-preventive agents in increasing androgen sensitivity of prostate cancer cells. An understanding of the mechanisms of AR gene

regulation may be of great importance in efforts to restore androgen responsiveness to the patients with androgen-independent prostate cancer, because this type of cancer is commonly unresponsive to most conventional therapies.

Acknowledgments

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References

- Feldman D, Malloy PJ, Gross C 1996 Vitamin D: metabolism and action. In: Marcus R, Feldman D, Kelsey J (eds) Osteoporosis. Academic Press, San Diego, pp 205-235
- Haussler MR, Whitfield GK, Haussler CA, Hsieh JC, Thompson PD, Selznick SH, Dominguez CE, Jurutka PW 1998 The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. *J Bone Miner Res* 13:325-349
- Walters MR 1992 Newly identified actions of the vitamin D endocrine system. *Endocr Rev* 13:719-764
- Bikle DD 1992 Clinical counterpoint: vitamin D: new actions, new analogs, new therapeutic potential. *Endocr Rev* 13:765-784
- Gross C, Peehl DM, Feldman D 1997 Vitamin D and prostate cancer. In: Feldman D, Glorieux FH, Pike JW (eds) Vitamin D. Academic Press, San Diego, pp 1125-1140
- van Leeuwen JPTM, Pols HAP 1997 Vitamin D: Anticancer and differentiation. In: Feldman D, Glorieux FH, Pike JW (eds) Vitamin D. Academic Press, San Diego, pp 1089-1106
- Baker AR, McDonnell DP, Hughes M, Crisp TM, Mangelsdorf DJ, Haussler MR, Pike JW, Shine J, O'Malley BW 1988 Cloning and expression of full-length cDNA encoding human vitamin D receptor. *Proc Natl Acad Sci USA* 85:3294-3298
- Skowronski RJ, Peehl DM, Feldman D 1993 Vitamin D and prostate cancer: 1,25-dihydroxyvitamin D₃ receptors and actions in human prostate cancer cell lines. *Endocrinology* 136:20-26
- Skowronski RJ, Peehl DM, Feldman D 1995 Actions of vitamin D₃ analogs on human prostate cancer cell lines: comparison with 1,25-dihydroxyvitamin D₃. *Endocrinology* 136:20-26
- Miller GJ, Stapleton GE, Ferrara JA, Lucia MS, Pfister S, Hedlund TE, Upadhyay P 1992 The human prostatic carcinoma cell line LNCaP expresses biologically active, specific receptors for 1 α ,25-dihydroxyvitamin D₃. *Cancer Res* 52:515-520
- Peehl DM, Skowronski RJ, Leung GK, Wong ST, Stamey TA, Feldman D 1994 Antiproliferative effects of 1,25-dihydroxyvitamin D₃ on primary cultures of human prostatic cells. *Cancer Res* 54:805-810
- Miller GJ, Stapleton GE, Hedlund TE, Moffatt KA 1995 Vitamin D receptor expression, 24-hydroxylase activity, and inhibition of growth by 1 α ,25-dihydroxyvitamin D₃ in seven human prostatic carcinoma cell lines. *Clin Cancer Res* 1:997-1003
- Hedlund TE, Moffatt KA, Miller GJ 1996 Vitamin D receptor expression is required for growth modulation by 1 α ,25-dihydroxyvitamin D₃ in the human prostatic carcinoma cell line ALVA-31. *J Steroid Biochem Mol Biol* 58:277-288
- Hedlund TE, Moffatt KA, Miller GJ 1996 Stable expression of the nuclear vitamin D receptor in the human prostatic carcinoma cell line JCA-1: evidence that the antiproliferative effects of 1 α ,25-dihydroxyvitamin D₃ are mediated exclusively through the genomic signaling pathway. *Endocrinology* 137:1554-1561
- Esquenet M, Swinnen JV, Heyns W, Verhoeven G 1996 Control of LNCaP proliferation and differentiation: actions and interactions of androgens, 1 α ,25-dihydroxycholecalciferol, all-trans retinoic acid, 9-cis retinoic acid, and phenylacetate. *Prostate* 28:182-194
- Hsieh T, Wu JM 1997 Induction of apoptosis and altered nuclear/cytoplasmic distribution of the androgen receptor and prostate-specific antigen by 1 α ,25-dihydroxyvitamin D₃ in androgen-responsive LNCaP cells. *Biochem Biophys Res Commun* 235:539-544
- Blutt SE, Allegretto EA, Pike JW, Weigel NL 1997 1,25-dihydroxyvitamin D₃ and 9-cis-retinoic acid act synergistically to inhibit the growth of LNCaP prostate cells and cause accumulation of cells in G1. *Endocrinology* 138:1491-1497
- Campbell MJ, Elstner E, Holden S, Uskokovic M, Koeffler HP 1997 Inhibition of proliferation of prostate cancer cells by a 19-nor-hexafluoride vitamin D₃ analogue involves the induction of p21waf1, p27kip1 and E-cadherin. *J Mol Endocrinol* 19:15-27
- Zhuang SH, Schwartz GG, Cameron D, Burnstein KL 1997 Vitamin D receptor content and transcriptional activity do not fully predict antiproliferative effects of vitamin D in human prostate cancer cell lines. *Mol Cell Endocrinol* 126:83-90
- Zhuang SH, Burnstein KL 1998 Antiproliferative effect of α ,25-dihydroxyvitamin D₃ in human prostate cancer cell line LNCaP involves reduction of cyclin-dependent kinase 2 activity and persistent G1 accumulation. *Endocrinology* 139:1197-1207
- Zhao XY, Ly LH, Peehl DM, Feldman D 1997 1 α ,25-dihydroxyvitamin D₃ actions in LNCaP human prostate cancer cells are androgen-dependent. *Endocrinology* 138:3290-3298
- Hsieh TY, Ng CY, Mallouh C, Tazaki H, Wu JM 1996 Regulation of growth, PSA/PAP and androgen receptor expression by 1 α ,25-dihydroxyvitamin D₃ in the androgen-dependent LNCaP cells. *Biochem Biophys Res Commun* 223:141-146
- Mizokami A, Yeh SY, Chang C 1994 Identification of 3',5'-cyclic adenosine monophosphate response element and other cis-acting elements in the human androgen receptor gene promoter. *Mol Endocrinol* 8:77-88
- Burton K 1956 A study of conditions and mechanisms of the diphenyl amine colorimetric estimation of deoxyribonucleic acid. *Biochem J* 62:315-323
- Malloy PJ, Hochberg Z, Pike JW, Feldman D 1989 Abnormal binding of vitamin D receptors to deoxyribonucleic acid in a kindred with vitamin D-dependent rickets, type II. *J Clin Endocrinol Metab* 68:263-269
- Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 72:248-254
- Zhao XY, Eccleshall TR, Krishnan AV, Gross C, Feldman D 1997 Analysis of vitamin D analog-induced heterodimerization of vitamin D receptor with retinoid X receptor using the yeast two-hybrid system. *Mol Endocrinol* 11:366-378
- Krishnan A, Feldman D 1997 Regulation of vitamin D receptor abundance. In: Feldman D, Glorieux FH, Pike JW (eds) Vitamin D. Academic Press, San Diego, pp 179-200
- Chen TL, Li JM, VanYe T, Cone CM, Feldman D 1986 Hormonal responses to 1,25-dihydroxyvitamin D₃ in cultured mouse osteoblast-like cells: modulation by changes in receptor level. *J Cell Physiol* 126:21-28
- Krishnan AV, Cramer SD, Bringham FR, Feldman D 1995 Regulation of 1,25-dihydroxyvitamin D₃ receptors by parathyroid hormone in osteoblastic cells: role of second messenger pathways. *Endocrinology* 136:705-712
- Mora GR, Mahesh VB 1996 Autoregulation of androgen receptor in rat ventral prostate: involvement of c-fos as a negative regulator. *Mol Cell Endocrinol* 124:111-120
- Radwan F, Leger F, Carmel M, Elhilali M, Lehoux J-G 1986 Characterization of androgen receptors in normal and malignant human prostatic tissues. *Prostate* 9:147-158
- Gong Y, Blok LJ, Perry JE, Lindzey JK, Tindall DJ 1995 Calcium regulation of androgen receptor expression in the human prostate cancer cell line LNCaP. *Endocrinology* 136:2172-2178
- Tetsuka M, Hillier SG 1996 Androgen receptor gene expression in rat granulosa cells: the role of follicle-stimulating hormone and steroid hormones. *Endocrinology* 137:4392-4397
- Shen R, Lin MC, Sadeghi F, Swerdloff RS, Rajfer J, Gonzalez-Cadavid NF 1996 Androgens are not major down-regulators of androgen receptor levels during growth of the immature rat penis [published erratum appears in *J Steroid Biochem Mol Biol* 58:137]. *J Steroid Biochem Mol Biol* 57:301-313
- Stubbs AP, Lalani EN, Stamp GW, Hurst H, Abel P, Waxman J 1996 Second messenger up-regulation of androgen receptor gene transcription is absent in androgen insensitive human prostatic carcinoma cell lines, PC-3 and DU-145. *FEBS Lett* 383:237-240
- DeFranco DB, Ramakrishnan C, Tang Y 1998 Molecular chaperones and subcellular trafficking of steroid receptors. *J Steroid Biochem Mol Biol* 65:51-58
- Lee C, Sutkowski DM, Sensibar JA, Zelner D, Kim I, Amsel I, Shaw N, Prins GS, Kozlowski JM 1995 Regulation of proliferation and production of prostate-specific antigen in androgen-sensitive prostatic cancer cells, LNCaP, by dihydrotestosterone. *Endocrinology* 136:796-803
- Kokontis J, Takakura K, Hay N, Liao S 1994 Increased androgen receptor activity and altered c-myc expression in prostate cancer cells after long-term androgen deprivation. *Cancer Res* 54:1566-1573
- Umekita Y, Hiipakka RA, Kokontis JM, Liao S 1996 Human prostate tumor growth in athymic mice: inhibition by androgens and stimulation by finasteride. *Proc Natl Acad Sci USA* 93:11802-11807
- Kokontis J, Hay N, Liao S 1998 Progression of LNCaP prostate tumor cells during androgen deprivation: hormone-independent growth, repression of proliferation by androgen, and role for p27^{kip1} in androgen-induced cell cycle arrest. *Mol Endocrinol* 12:941-953
- Yuan S, Trachtenberg J, Mills GB, Brown TJ, Xu F, Keating A 1993 Androgen-induced inhibition of cell proliferation in an androgen-insensitive prostate cancer cell line (PC-3) transfected with a human androgen receptor complementary DNA [published erratum appears in *Cancer Res* 55:719]. *Cancer Res* 53:1304-1311
- Zhou HY, Chang SM, Chen BQ, Wang Y, Zhang H, Kao C, Sang QA, Pathak SJ, Chung LW 1996 Androgen-repressed phenotype in human prostate cancer. *Proc Natl Acad Sci USA* 93:15152-15157

factors and cell adhesion molecules, many of which also contribute to prostate carcinogenesis, have been identified. Moreover, it is now accepted that the prostate is an endocrine gland capable to synthesize and secrete a wide variety of hormones. It has been reported that gonadotropins are expressed in the human prostate (Br J Cancer 1990, 61:225). In this study we analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) the mRNA expression for FSH receptor in human prostate. Prostate tissue was obtained from a total of 18 patients (age 55-79 years). Each sample was evaluated histologically. Four patients were affected by benign prostatic hyperplasia (BPH) and 14 by adenocarcinoma. Specimens of normal tissue were obtained in four patients with carcinoma from the portion of the gland not affected by the tumor. The samples were quickly frozen and kept at -80 °C. Total RNA was extracted and RT-PCR performed on equal amounts of RNA samples. Specific primers for exon 10 of the FSH receptor gene were used. The reaction was carried out in 50 µl, after an initial denaturation for 3 min at 94 °C, for 40 cycles of denaturation (94 °C, 1 min), annealing (55 °C, 1 min) and extension (72 °C, 1 min), followed by a final elongation of 5 min at 72 °C. Control PCR reactions were performed on RNA samples before reverse transcription to confirm the absence of genomic DNA contamination. PCR products were resolved and visualized on 1.5% agarose gels stained by ethidium bromide. The results obtained show that the FSH receptor mRNA is expressed in human prostate. All the normal tissues examined showed low levels of expression. Two out of four BPH and eight out of fourteen adenocarcinoma were positive, showing higher levels of expression of the gene compared to normal, with a wide degree of variability between the samples. No correlation with histological Gleason grading of the adenocarcinoma specimens was found. The expression of the FSH receptor in human prostate and its up regulation in some cases of BPH and carcinoma suggests that FSH may play a role in the development of some prostatic disorders, as a factor that takes over androgen regulation of prostatic cell proliferation during the development of androgen-independent growth. Moreover, the recently reported down regulation of the inhibin-α gene expression in prostate cancer (J Clin Endocrinol Metab 1998, 83:969) supports the concept of an autocrine/paracrine regulation of FSH action within the prostate.

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DIFFERENT EXPRESSION OF ESTROGEN RECEPTOR α AND β IN PRIMARY CULTURES OF NORMAL AND MALIGNANT HUMAN PROSTATE EPITHELIAL CELLS.

A. A. Sinisi,*¹ D. Pasquali,¹ V. Rossi,¹ C. Abbondanza,² G. A. Puca,² A. Bellastella.¹

¹Endocrinology, ²General Pathology, Second University of Naples, Naples, Italy

The estrogen receptor α (ERα) has been localized to the stroma and basal epithelial cells of human prostate. Recently an high level of expression of a novel ER subtype, ERβ, has been demonstrated in rat prostate epithelium, but the presence and the role of this subtype in human prostate is unknown. In this study we investigated the expression ER α and β genes and screened the presence of receptor proteins in normal (N) and malignant (K) prostate epithelial cell (EC) primary cultures. **Methods:** Primary epithelial cell cultures were established in SFM-keratinocyte medium (Gibco-BRL) with growth factors and 5% FCS, after collagenase digestion of minced N and K prostate surgical samples. mRNA transcripts of ER α and β were detected by RT-PCR using specific primers amplifying a region comprise between exon 4 and 6 (set 1). ER α expression was further studied using a set of primers comprised between exon 6 and exon 8 (set 2). Receptor proteins were searched in cell lysate by Western blotting (WB) using a monoclonal antibody raised against a C-terminal epitopes common to ER α and β, and another raised against N-terminal part of ER α. **Results:** In NEC both ER α and β genes were expressed. In KEC only ER α mRNA was clearly detected when amplified with set 1. By WB, using the C-terminus directed antibody, the ER α and β proteins were found in NEC and KEC. **Conclusions:** We demonstrated the presence of ER α and β gene transcripts and relative proteins in cultured EC from normal human prostates. In KEC ERβ mRNA and protein were undetectable, and ER α gene resulted translated in a protein probably changed in C-terminal domain.

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INHIBITION OF HUMAN PROSTATE CANCER CELLS GROWTH BY ANTIOXIDANTS IS MEDIATED THROUGH APOPTOSIS.

Kushlani Gunawardena,* Darrell K. Murray, Wayne A. Meikle. Medicine & Pathology, University of Utah, Salt Lake City, UT

Introduction. Many human prostate cancer cells have escaped the apoptotic effects of natural regulators of cell growth such as transforming growth factor β1 (TGFβ-1) and tumor necrosis factor. Some evidence suggests that antioxidants might suppress the development of prostate cancer.

Methods. To investigate if prostate cancer cell growth can be influenced by apoptotic activators, DU 145 (androgen unresponsive), ALVA 101 (moderately androgen responsive) and LNCaP (androgen responsive) were grown in RPMI 1640 medium supplemented with bovine calf serum and antibiotics and were treated with various antioxidants for 1-7 days. Cell growth was then determined with the Cell Titer 96 AQ assay (Promega). Apoptosis was assessed by cell death detection ELISA assay (quantitating histone-associated DNA fragments) in the cytoplasm of cells, and cell viability by methylene blue exclusion.

Results. (±)-α-Tocopherol (vitamin E) treatment for 1-7 days at concentrations of 0.08-5 µg/mL modestly affected growth compared to other antioxidants tested. Tocopherol produced a significant (p<0.01) inhibition of ALVA 101 and LNCaP (10% inhibition compared to control; 2.5 µg/mL; at 6-7 days; n=6), but did not show a dose response inhibition. DU 145 cells were not growth inhibited with tocopherol treatment. However,

pyrrolidinedithiocarbamate (PDT) produced a significant (p<0.01, n=6) dose response inhibition (20-80% inhibition) of DU 145 and ALVA 101 cells and a significant (p<0.01) inhibition of LNCaP cells without a dose response relationship also at concentrations of 2.5-20 µg/mL. When cells were grown to confluence, PDT failed to inhibit cell growth. A third compound, diethyldithiocarbamic acid (DETC), incubated for 1-7 days also produced significant dose response suppression of cell growth of DU 145 and ALVA 101 cells (p<0.01; 20-80% inhibition; n=6) at concentrations of 1.25-80 µg/mL. LNCaP cells were inhibited (p<0.01; 28% inhibition; n=6) by DETC, but they did not show a dose response inhibition compared to the other two cell lines. ELISA assay of nucleosomes obtained from ALVA 101 cells treated with PDT documented significant (p<0.03; n=6) apoptosis compared to untreated controls.

Conclusions. These results demonstrate that antioxidants modulate human prostate cancer cell proliferation by altering apoptosis, but they do not cause necrosis or apoptosis of confluent cells.

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ANDROGEN INSENSITIVITY DUE TO A DOUBLE MUTATION IN THE ANDROGEN RECEPTOR OF A NEW HUMAN PROSTATE CANCER CELL LINE MDA PCa 2a.

Xiao-Yan Zhao,* Bryan Boyle,¹ Aruna V Krishnan,¹ Peter J Malloy,¹ Nora M Navone,³ Donna M Peehl,² David Feldman.¹ ¹Medicine, Stanford University, Palo Alto, CA, ²Urology, Stanford University, Palo Alto, CA, ³GU Medical Oncology, MDACC, Houston, TX

We have characterized the androgen receptor (AR) in a new human prostate cancer cell line, MDA PCa 2a, that has recently been established from a bone metastasis of a patient whose cancer was exhibiting androgen-independent growth. These cells express abundant AR (Nmax=685±149 fmol/mg protein), as determined by equilibrium binding assays with [³H]DHT. However, Scatchard analyses showed the AR binding affinity for DHT in these cells to be only 25 nM, 50-fold lower than the AR in LNCaP cells (Kd=0.5 nM) or the wildtype AR in MCF-7 cells (Kd=0.4 nM). DNA sequencing analyses of the AR gene in MDA PCa 2a cells revealed two mutations in the ligand-binding domain, L701H and T877A, the latter being reported previously in the LNCaP cells. Compared to LNCaP, the new cell line is 10 to 1000-fold less responsive to androgens in cell growth assays as well as in stimulation of PSA. Interestingly, in the absence of added androgens, the new cell line expresses 15-fold higher baseline levels of PSA than LNCaP, suggesting constitutive expression of its PSA protein. We are currently recreating both mutations by *in vitro* mutagenesis and evaluating the recreated mutant AR in terms of its ligand binding affinity, specificity, and transactivation properties. In summary, we have identified two mutations in the AR gene of MDA PCa 2a cell line that are likely responsible for the decreased binding affinity for DHT and the partial androgen insensitivity observed in these cells. Thus, this new cell line can serve as a functionally relevant model system of advanced prostate cancer with bone metastases.

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SOMATOSTATIN AFFECTS THE LEVELS OF TOPOISOMERASE II GENE AND PROTEIN IN LNCaP CELLS.

T.A.L. Brevini,* L.A. Favetta, Faviana Cillo, Chiara Agradi, Marcella Motta. Institute of Endocrinology, University of Milan, Milan, Italy

Somatostatin (S) has been previously shown to exert an inhibitory effect on the growth of the human prostatic cancer cell line LNCaP (Lymph Node Carcinoma of the Prostate). However, the molecular mechanisms, possibly involved in the hormone antiproliferative action remain still elusive and have to be further elucidated. We recently reported the effect of S on LNCaP cell gene transcription, using RNA differential display (dd)PCR techniques. In particular we identified up-regulated levels of expression of a 170 cDNA fragment in LNCaP cells exposed to S. The interaction of the hormone with the present transcript was further confirmed by both Northern Analysis and Ribonuclease Protection Assay. Comparison of its cDNA sequence with the Genebank and EMBL databases revealed a 91% degree of homology with the deposited sequence of the nuclear enzyme Topoisomerase II. To investigate the physiological impact of this finding we evaluated the effect of the hormone on the corresponding protein. To this purpose we cultured LNCaP cells either in the presence or in the absence of S. We then extracted LNCaP cell constitutive proteins and separated them on a 8-15% SDS PAGE. Proteins were blotted onto Nitrocellulose filters and probed for the levels of Topoisomerase II protein using a specific monoclonal antibody. The results obtained indicate that S interacts with both the levels of transcription and the concentration of Topoisomerase II protein when used at the same dose shown to exert an antiproliferative effect on LNCaP cells.

This enzyme is a key molecule regulating cell cycle and, more specifically, chromosome condensation and mitosis. Its levels as well as its timing of expression are considered to be crucial for the correct completion of cell cycle. Thus, alteration of these parameters may lead to an uncoordinated replication cycle.

The observation that S probably enhances the levels of Topoisomerase II molecule suggests the possibility that the hormone might affect LNCaP cell proliferation directly interfering with this enzyme and, therefore, with the correct cell cycle dynamic in LNCaP cell line.

(Supported by AIRC and by MURST).

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mechanism of action of AA, the effect of AA on PKC activity was examined by adding inhibitors of PKA (H8 and H89), lipoxygenase (NDGA and esculetin), or cyclooxygenase (indomethacin) to the cultures in the presence or absence of 24,25. The identification of which PKC isoform is activated by AA was determined by PKC isoform-specific antibodies. AA stimulated [³H]-thymidine incorporation and inhibited the activity of alkaline phosphatase and PKC, but had no effect on matrix production. In contrast, 24,25 inhibited [³H]-thymidine incorporation and stimulated alkaline phosphatase and PKC as well as matrix production. In cultures treated with both agents, the effects of 24,25 were abrogated by AA. Studies using cyclooxygenase and lipoxygenase inhibitors indicated that the effects of AA were due in part to prostaglandins but not leukotrienes. AA did not alter the translocation of PKC from the cytosol to the membrane. Direct addition of AA to isolated matrix vesicles activated PKC, while addition to plasma membranes inhibited PKC. This was opposite to the effect seen with 24,25; in addition, when added to the membranes at the same time, AA abrogated the effect of 24,25. Studies with PKC isoform-specific antibodies indicated that the predominant PKC isoform affected by AA treatment was PKC α . This study shows that AA regulates RC proliferation, differentiation, and matrix production. The effect of AA on the cultures was the opposite of that observed with 24,25. We conclude that 24,25 mediates its effects on RC cells by inhibiting AA production. AA also had a direct effect on membranes isolated from the cultures, suggesting that there may be a unique membrane receptor for AA.

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Liarozole Acts Synergistically With 1,25-Dihydroxyvitamin D3 to Inhibit DU 145 Human Prostate Cancer Cell Growth by Blocking 24-Hydroxylase Activity. X. Y. Zhao, Lan H. Ly, Leah Holloway, David Feldman, Department of Medicine, Stanford University, Stanford, CA.

1 α ,25-Dihydroxyvitamin D3 [1,25-(OH)₂D₃] inhibits the proliferation of many cancer cells in culture but not the aggressive human prostate cancer cell line DU 145. We postulated that the 1,25-(OH)₂D₃-resistant phenotype of DU 145 cells might result from the high levels of expression of 25-hydroxyvitamin D-24-hydroxylase (24-hydroxylase) induced by treatment with 1,25-(OH)₂D₃. Since this P450 enzyme initiates 1,25-(OH)₂D₃ catabolism, we presumed a high level of enzyme induction could limit the effectiveness of the 1,25-(OH)₂D₃ antiproliferative action. To examine this hypothesis we explored combination therapy with liarozole fumarate (R85,246), an imidazole derivative currently in trials for prostate cancer therapy. Since imidazole derivatives are known to inhibit P450 enzymes, we postulated that this drug would inhibit 24-hydroxylase activity, increasing 1,25-(OH)₂D₃ half-life, thereby enhancing 1,25-(OH)₂D₃ antiproliferative effects on DU 145 cells. Cell growth was assessed by measurement of viable cells using the MTS assay. Neither 1,25-(OH)₂D₃ (1-10 nM) nor liarozole (1-10 μ M) inhibited DU 145 cell growth when given alone. However, together at 4 days, 1,25-(OH)₂D₃ (10 nM)/liarozole (1 μ M) inhibited growth 65%. We used a thin layer chromatography method to assess 24-hydroxylase activity and demonstrated that liarozole (1-100 μ M) inhibited this P450 enzyme in a dose-dependent manner. Moreover, liarozole treatment caused a significant increase in 1,25-(OH)₂D₃ half-life from 11 to 31 h. In addition, 1,25-(OH)₂D₃ can cause homologous up-regulation of the VDR and in the presence of liarozole, this effect was augmented thus enhancing 1,25-(OH)₂D₃ activity. Western blot analyses demonstrated that DU 145 cells treated with 1,25-(OH)₂D₃/liarozole showed greater VDR up-regulation than with either drug alone. In summary, our data demonstrate that liarozole augments the ability of 1,25-(OH)₂D₃ to inhibit DU 145 cell growth. The mechanism appears to be due to inhibition of 24-hydroxylase activity leading to increased 1,25-(OH)₂D₃ half-life and augmentation of homologous up-regulation of VDR. We raise the possibility that combination therapy using 1,25-(OH)₂D₃ and liarozole or other inhibitors of 24-hydroxylase, both in non-toxic doses, might serve as an effective treatment for prostate cancer.

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Expression of 11 β -Hydroxysteroid Dehydrogenase Type 2 in Rat Osteosarcoma Cells: Autocrine Regulation of Glucocorticoid Responses in Bone. M. Hewison, L. J. Eyre,* R. Bland,* M. C. Sheppard,* P. M. Stewart,* Department of Medicine, The University of Birmingham, Birmingham, UK.

The enzyme 11 β -hydroxysteroid dehydrogenase (11 β -HSD) acts as a pre-receptor signaling mechanism for corticosteroids, by regulating the access of active glucocorticoids to both glucocorticoid (GR) and mineralocorticoid receptors (MR). To examine a possible relationship between endogenous glucocorticoid metabolism and osteoblast activity, we have characterized the expression of 11 β -HSD isozymes in rat osteosarcoma cells. Analysis of mRNA from ROS 25/1, UMR106 and ROS 17/2.8 cells revealed transcripts for both 11 β -HSD type 1 (11 β -HSD1) and type 2 (11 β -HSD2) in all three cell lines. However, enzyme activity studies showed only high affinity (K_m = 45 nM) dehydrogenase activity (inactivation of corticosterone to 11-dehydrocorticosterone), characteristic of 11 β -HSD2. Conversion of corticosterone to 11-dehydrocorticosterone in ROS 25/1 > UMR106 > ROS 17/2.8 cells. Activity in all three cell lines was up-regulated in an autocrine fashion following treatment with natural glucocorticoids, but not dexamethasone (dex). Induction of 11 β -HSD2 was unaffected by co-treatment with the GR antagonist RU486 or the MR antagonist RU752, but was completely inhibited by treatment with 11 β -HSD inhibitor glycyrrhetic acid. Analysis of

³H-dex binding revealed approximately 60,000 GR/cell in all three cell lines, with much lower levels of MR detected using ³H-aldoosterone (aldo). Parallel studies of alkaline phosphatase activity indicated that dex and corticosterone stimulated activity in ROS 17/2.8 cells but had no effect on ROS 25/1 cells or UMR106; the classical MR ligand, aldo, was without effect in all three cell lines. Up-regulation of alkaline phosphatase activity in ROS 17/2.8 by dex was completely inhibited by RU486 whereas RU752 had no effect. In contrast, induction of alkaline phosphatase activity by corticosterone was inhibited by both RU486 and RU752, suggesting different receptor mechanisms for osteoblast responses to synthetic versus naturally occurring glucocorticoids. These studies demonstrate capacity for local inactivation of glucocorticoids in osteoblasts. The presence of GR and lack of functional response to aldo suggest that 11 β -HSD2 in rat osteoblasts acts in a protective fashion by regulating GR occupancy; cells with relatively high levels of 11 β -HSD2 activity (ROS 25/1 and UMR106) were insensitive to glucocorticoids, whilst cells with low levels demonstrated functional responses to both dex and corticosterone. We therefore postulate that pre-receptor regulation of ligand availability is a major determinant of glucocorticoid effects on bone.

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Glucocorticoids Inhibit the Expression of Hepatocyte Growth Factor/Scatter Factor (HGF/SF) in Osteoblasts. Frederic Blanquart, Ernesto Canalis, Saint Francis Hospital and Medical Center, Hartford, CT.

Glucocorticoids have profound effects on skeletal metabolism, and delay wound and possibly fracture healing. However, the mechanisms involved are not known. Hepatocyte growth factor/scatter factor (HGF/SF) plays a central role in tissue regeneration and healing and enhances the replication of cells of the osteoblastic lineage. Expression of HGF/SF in osteoblasts is limited, but it is enhanced by factors that accelerate fracture repair, confirming the role of HGF/SF in healing. We examined the effects of cortisol on HGF/SF expression in osteoblast enriched cells from 22 day fetal rat calvariae (Ob cells). Cortisol at 1 μ M for 24 to 48 h decreased the basal expression of HGF/SF transcripts in Ob cells by approximately 50%. In contrast, fibroblast growth factor (FGF) -2 at 2 nM and platelet derived growth factor (PDGF) BB at 3 nM for 24 h significantly increased HGF/SF transcripts by 2 to 8 fold in osteoblast cultures. Cortisol at 1 μ M significantly decreased the stimulatory effect of FGF-2 and PDGF BB on HGF/SF expression in Ob cells. An analogous effect was observed in the MC3T3 osteoblastic cell line. Cortisol also caused a time and dose dependent increase in the level of expression of c-met, a protooncogene encoding for the HGF/SF receptor. This effect was observed at cortisol concentrations of 10 to 1,000 nM and after 6 to 24 h cortisol increased c-met transcripts by 3 fold. In conclusion, FGF-2 and PDGF BB increase HGF/SF mRNA expression, and cortisol decreases basal and growth factor-induced HGF/SF transcripts. This effect may be critical to the inhibitory actions of glucocorticoids on fracture healing. Cortisol induction of c-met mRNA levels may be a compensatory mechanism to maintain HGF/SF function in osteoblasts in conditions of glucocorticoid excess.

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Calreticulin Regulates Integrin $\alpha_v\beta_3$ Response To 1 α ,25(OH)₂D₃. Zhijie Chang,¹ K. A. Hruska,² S. Dedhar, S. L. Teitelbaum,² F. P. Ross,² Xu Cao.¹ ¹Department of Pathology, University of Alabama, Birmingham, AL, ²Washington University, St. Louis, MO.

1 α ,25(OH)₂D₃ plays an important role in osteoclast differentiation. Calreticulin, a calcium-binding protein, can activate gene transcription such as $\alpha_v\beta_3$. 1 α ,25(OH)₂D₃ transactivation activity is modulated by calreticulin, which binds to the DNA binding domain of 1 α ,25(OH)₂D₃ receptor.

In this study we demonstrated that calreticulin dose-dependently inhibited both VDR binding and its transactivation by transfection of integrin β_3 and VDRE and by gel-shift. Further more, we showed that over-expression of calreticulin blocks 1 α ,25(OH)₂D₃-induced integrin β_3 mRNA level by Northern blot analysis. Whereas, the over-expression of anti-sense calreticulin cDNA enhanced β_3 mRNA levels in HD11 cells treated with 1 α ,25(OH)₂D₃. To further examine the function of calreticulin in osteoclast differentiation, we constructed calreticulin-adenovirus expression vector which can express calreticulin at very high level in transfected osteoclast like cells. The bone resorption and cell attachment was inhibited in the osteoclast precursors infected with adenovirus bearing calreticulin, compared with cells infected with adenovirus alone. Finally, we use immunostaining assay to demonstrate that calreticulin was involved in VDR translocation into nucleus. In summary, we conclude that calreticulin inhibits integrin β_3 expression by direct interacting with VDR, and affects attachment and bone resorption mediated by osteoclasts.